Cloning and Characterization of β Integrin Subunits in Sea Urchin Embryos

by

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Abstract

In the sea urchin embryo, cell rearrangements occurring during gastrulation are involved in the establishment of cell fates. The integrins are a family of cell adhesion molecules that play essential roles in the morphogenetic events taking place during vertebrate embryogenesis, and are therefore likely candidates for mediators of cell adhesion during sea urchin gastrulation.

Sea urchin embryo cells adhere and spread on an artificial substrate (Pronectin-F) that contains a conserved integrin binding motif, GRGDS. Spreading, but not adhesion, can be inhibited with soluble GRGDS peptides indicating that this interaction is specific. Antibodies raised against adherent cells suggest that cells attaching to Pronectin-F are of epithelial origin. One of the antibodies 8F2 recognizes a cell surface epitope that is localized to the margins of all epithelial cells in the early embryo. The domain that cross-reacts with 8F2 becomes restricted to the oral face and developing digestive tract by the prism stage.

Using degenerate primers in RT PCR, three novel β integrin subunits have been isolated from sea urchin embryos. The predicted amino acid sequences of these subunits bear 40% similarity to vertebrate integrins, and contain highly conserved ligand binding domains. Amino acid sequence comparisons of the sea urchin β integrin to known β integrins indicates that the sea urchin molecules represent novel forms of β integrin subunits. The three sea urchin subunits are expressed as maternal 7.5 Kb transcripts. The βC (cleavage) subunit peaks in expression during cleavage and decreasing levels of the transcript are detectable up until the gastrula stage. The βG (gastrulation) subunit is detectable in all stages of development, but peaks in expression during gastrulation. The βL (larval) subunit is expressed at low levels up until the late gastrula when high levels of expression are detected through until the pluteus stage. *In situ* localization of the βG

subunit indicates that most cells of the embryo express this molecule. High levels of expression are detected in the primary mesenchyme cells, the developing gut, the pigment cells, and in the oral ectoderm after the end of gastrulation. *In situ* localization of the βL subunit indicates that it is expressed in the secondary mesenchyme up until these cells detach from the tip of the archenteron. Once the secondary mesenchyme cells have migrated into the blastocoel they no longer express βL. The βL transcript is also evident in the primary mesenchyme cells, as well as a number of blastocoelar cells, from the midgastrula stage onwards. Antisera raised against expressed fragments of the βG and βL subunits recognize 120 Mr proteins on western blots. The developmental regulation of translation mimics the transcriptional regulation of βG and βL. The localization and temporal expression of these β integrin subunits suggests that these molecules mediate diverse cell adhesion events that are active during gastrulation.

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I am not grouchy.

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%	percent
° C	degrees centigrade
µg	microgram
µjoules	microjoules
µl	microlitre
µm	micrometre
3'	three prime
5'	five prime
AA	amino acid
AEBSF	aminoethylbenzene-sulfonylflouride
BCIP	5-Bromo-4-chloro-3-indoyl-phosphate
bp	base pair
BSA	bovine serum albumin
C-terminal	carboxy-terminal
C. elegans	*Caenorhabditis elegans*
cDNA	copy deoxyribonucleic acid
cm	centimetre
CMFSW	calcium magnesium free sea water
cRNA	copy ribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytadine triphosphate
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
Drosophila	*Drosophila melanogastor*
dT	deoxythymadine
DTT	dithiothritol
dTTP	deoxythymadine triphosphate
E. coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(-β-amino-ethyl ester) N,N'-tetra-acetic acid
ETOH	ethanol
Fab	fragment having the antigen binding site
FSW	filtered sea water
GRGDS	glycine-arginine-glycine-aspartic acid-serine
I-CAM	intercellular adhesion molecule
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton

LB	Luria-Bertani
M	molar
ml	millilitre
mm	millimetre
mM	millimolar
MMLV	moloney murine leukemia virus
N-CAM	neural cell adhesion molecule
N-terminal	amino-terminal
NEB	New England Biolabs
ng	nanogram
OD	optical density
ON	over night
PBS	phosphate bufferred saline
PBST	phosphate bufferred saline, 0.1% Tween-20
PCR	polymerase chain reaction
PF	postfertilization
PFU	plaque forming units
PMC	primary mesenchyme cell
PolyA+	polyadenylated
RGD	arginine-glycine-aspartic acid
RGES	arginine-glycine-glutamic acid-serine
RNA	ribonucleic acid
Rnase	ribonuclease
Rnasin	human placental ribonuclease inhibitor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide electrophoresis
SM	suspension medium
SMC	secondary mesenchyme cell
SSPE	standard sodium phosphate EDTA
SSW	sterile sea water
TAE	tris acetate EDTA
Taq	*Thermous aquaticus*
TBE	tris borate EDTA
TBS	tris bufferred saline
TCA	trichloroacetic acid
TE	tris EDTA
UV	ultraviolet
V-CAM	vascular cell adhesion molecule
X-Gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
Xenopus	*Xenopus laevis*

INTRODUCTION

1.0 Pattern formation and the development of embryonic form

During the late 1800's the experiments of Wilhelm Roux (translated into English in Willier and Oppenheimer, 1974) attempted to address the mechanisms by which cells acquire identity during early development. These experiments set the stage for others who approached development as a sequential process through which the complex form of the embryo is progressively elaborated from the fertilized egg. Today, we pursue many of the same questions, though it is now intuitive that the cells that arise through cleavage acquire differences and specializations through regulated gene expression. The early regional specialization of cells in an embryo is known as pattern formation (Davidson, 1994). In multicellular organisms, pattern formation results in a spatial or positional identity being established within a temporal framework. The spatial fields that are established during pattern formation events can often be correlated, through cell lineages, to specific cell fates. It has become increasingly evident that most early embryonic cells have the potential to acquire many fates and that cell fate is restricted as development procedes. As such, one cannot isolate the events that establish cell identity from those processes that define cell identity. The application of molecular techniques to the mechanisms that operate during embryogenesis provides the possibility of gaining an understanding of the processes establishing cell fate.

2.0 The role of cell movements during morphogenesis

While different organisms have diverse modes of development, there is a point in development observed in all organisms at which relative cell positions are highly predictable. It is during this time that early cell lineages are being defined (Davidson, 1990). It is apparent that lineage and pattern cannot be the only mechanisms by which cell fate is established, as certain phases of development are characterized by extensive rearrangements within the embryo. For instance, in *Drosophila* embryos, patterns that define the embryonic axes, mesoderm, and ectoderm are established and elaborated before cellularization of the blastoderm, and hence any possibility of morphogenetic movements (St. Johnston and Nüsslein-Volhard, 1992). However, the normal formation of appendages relies upon the morphogenetic rearrangement and recombination of previously established patterns within imaginal discs (Ingham and Martinez-Arias, 1992). The other extreme seems to occur in vertebrate embryos in which patterns originate from organizational centers (Kessler and Melton, 1994). In these embryos the patterns that define the three primary germ layers and the neural ectoderm are established by local influences during or after morphogenetic movements. Thus, in contrast to the situation observed in *Drosophila*, the highly directed and often predictable movements of cells observed in early vertebrate development are critical in establishing pattern rather than modifying previously established patterns.

Despite the crucial role that morphogenesis plays in development, the diversity of mechanisms mediating cell rearrangements is limited. Cells move either as cohesive sheets or as individuals (mesenchyme). From embryological studies it is evident that

morphogenetic movements are directed and that cellular rearrangements are not random. While the processes that guide cells during these rearrangements remain unclear, the molecules that mediate cell adhesion and migration are becoming characterized. Compared to the molecules that define cell identity, there are relatively few known families of cell surface molecules that modulate cell adhesion. This is not completely unexpected, as the mechanisms mediated by these molecules are few in number. Despite the limited mechanisms mediated by a small number of cell adhesion molecules, the consequences of these adhesive interactions are diverse.

It would seem to be contradictory for receptors to have limited ligand repertoires, yet mediate a great diversity of downstream events. This issue is made even more complex by the observation that similar ECM receptors binding identical ligands are expressed in diverse cell types and mediate diverse events (Adams and Watt, 1993). The ability of a cell to respond to receptor occupancy in a multitude of ways appears to be due to the interpretation of the signal. This may stem from spatially restricted presentation of ligands, or through temporal modulation of a cells ability to respond to the presence of a specific ligand. Central to this issue is that the ligands that support cell adhesion do not merely act as a scaffold for cell attachment, but must also have the ability to provide instructive influences to cells, and as such, signalling events must be transmitted across the cellular membrane. There are a number of families of transmembrane cell adhesion receptors that have been implicated in events such as those described above.

3.0 Transmembrane receptors active in development

Transmembrane adhesion molecules are receptors that bind ligands presented either as components of the ECM or as molecules on the surface of other cells. The receptors are presently classified into four major groups. The cadherins, a family of calcium dependant cell-cell adhesion molecules (reviewed by Takeichi 1991), which mediate homophilic interactions and as such regulate cell sorting events (Nose *et al.*, 1988). The role that cadherins play in developmental processes is the maintenance of epithelial sheets (Kintner 1992). The second major group are the immunoglobulin superfamily, which are divalent cation dependant receptors with considerable variation in structure. Some of these molecules, such as N-CAM, act in a homophilic fashion (Santoni *et al.*, 1989), while other molecules such as ICAM and VCAM act in heterophilic cell/cell adhesion events in association with another family of receptors, the integrins (Elices *et al.*, 1990). The selectins represent the third major group of cell adhesion molecules, and are a family of carbohydrate binding molecules identified on endothelial cells and leukocytes (Bevilaqua *et al.*, 1991). The selectins act in concert with other cell adhesion molecules to mediate dynamic adhesive events (Osborn 1990). The fourth recognized group are the integrins.

4.0 The Integrin family of receptors

The integrins are transmembrane, heterodimeric molecules composed of the non-covalent association of an α and β subunit. The ligands bound by integrins are found within the ECM, as well as being presented on the surface of other cells. The

transmembrane nature of the receptor suggests a link between the cytoplasm and the extracellular environment and there is now evidence that integrins function in both inside-out and outside-in signalling events.

4.2 Classification of integrins

The classification of integrins is complicated due to the multiple associations of various subunits, multiple ligand recognition by individual receptors, and subunit complexity produced through alternative exon splicing. Despite this, there are a number of generalities that emerge and can be used to classify integrins. The present classification scheme is based upon the associations between the eight known subclasses of β and 14 subclasses of α subunits (Hynes 1992). While the potential number of integrins resulting from the random association of these subunits is greater than 100, only 20 combinations have been identified (Figure 1, reviewed in Hynes 1992). Most of the α subunits only form an association with a single β subunit, although the αv subunit forms functional receptors with five different β subunits and as a consequence it's ligand binding repertoire is diverse. The β1 subunit also forms associations with a large number of α subunits and this is reflected in its ubiquitous distribution in most tissues. While the associations outlined in Figure 1 appear to hold true, new integrin subunits are being discovered and the classification scheme may have to be expanded or altered. It should also be noted that a number of invertebrate integrin subunits have been cloned and do not fall easily into the classification scheme described above.

Figure 1: Classification of integrins.

The known interactions between α and β subunits are indicated by line. The ligands of individual receptors, if known, are indicated to the left of the figure. Figure is after that in Integrins: Molecular and Biological Responses to the Extracellular Matirix. D. A. Cheresh and R. P. Meecham, eds., 1994.

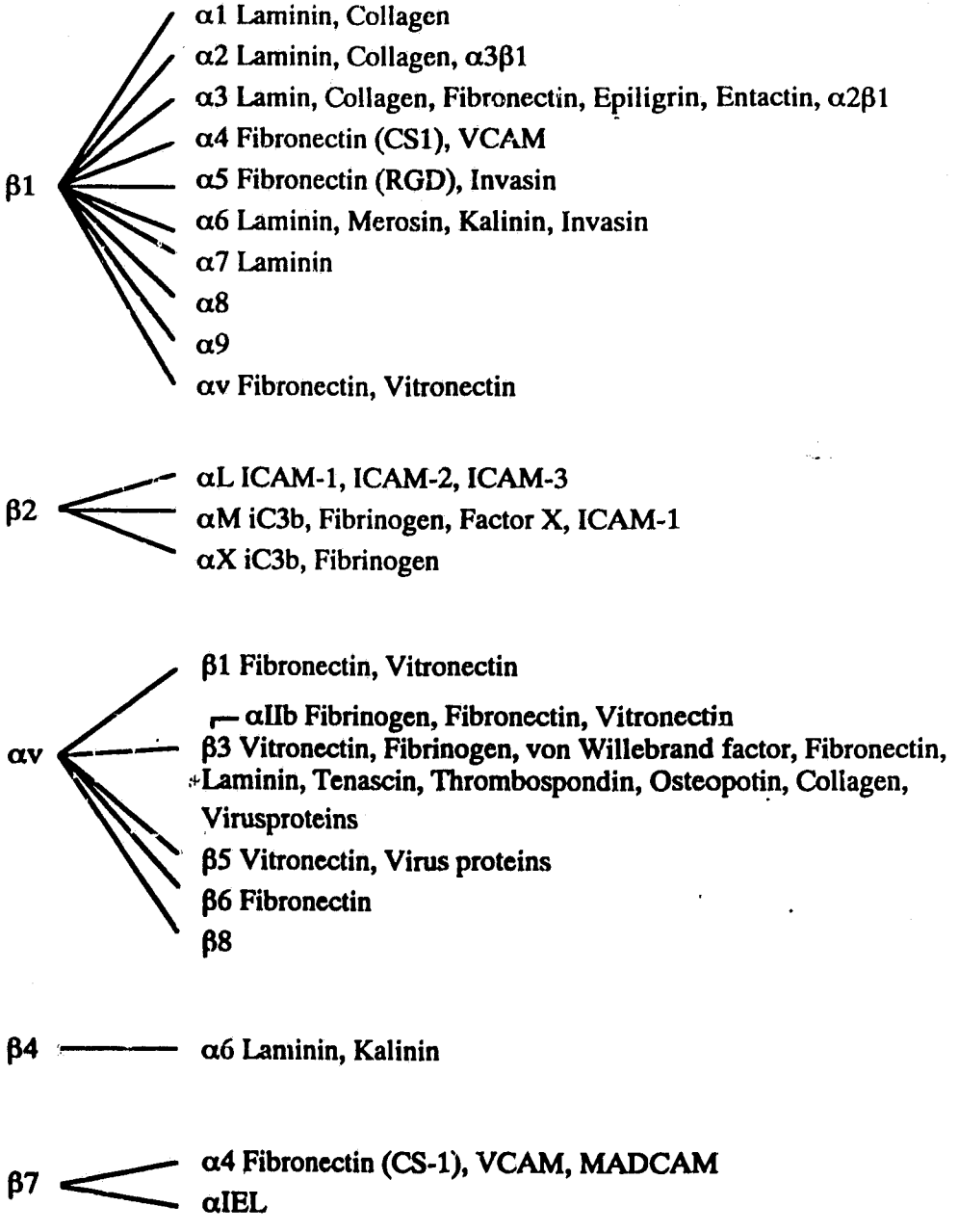

β1
α1 Laminin, Collagen
α2 Laminin, Collagen, α3β1
α3 Lamin, Collagen, Fibronectin, Epiligrin, Entactin, α2β1
α4 Fibronectin (CS1), VCAM
α5 Fibronectin (RGD), Invasin
α6 Laminin, Merosin, Kalinin, Invasin
α7 Laminin
α8
α9
αv Fibronectin, Vitronectin
β2
αL ICAM-1, ICAM-2, ICAM-3
αM iC3b, Fibrinogen, Factor X, ICAM-1
αX iC3b, Fibrinogen
αv
β1 Fibronectin, Vitronectin
αIIb Fibrinogen, Fibronectin, Vitronectin
β3 Vitronectin, Fibrinogen, von Willebrand factor, Fibronectin, Laminin, Tenascin, Thrombospondin, Osteopotin, Collagen, Virusproteins
β5 Vitronectin, Virus proteins
β6 Fibronectin
β8
β4
α6 Laminin, Kalinin
β7
α4 Fibronectin (CS-1), VCAM, MADCAM
αIEL

Table 1: Integrin classification and binding motifs.

Abreviations used in ligand column of Table 1: FN fibronectin, LN laminin, KN kalinin, VN vitronectin, COL collagen, FG fibrinogen, TSP thrombospondin, TN tenascin, OP osteopotin, vWF vonWillebrand factor, iC3b C3b component of complement, EP epilligrin, ET entactin.

Abbreviations in Motif column of Table 1 are standard single letter amino acid codes. The conserved aspartic acid residue is highlighted in the binding motif column.

Information for Table 1 is from, Intergins: Molecular and Biological Responses to the Extracellular Matrix. D. A. Cheresh and R. P. Meecham eds. Academic Press, San Diego. 1994. and Hynes 1992.

Subunits		Ligands	Motif
β1	α1	COL, LN	
	α2	COL, LN, α3β1	DGEA
	α3	FN, LN, COL, EP, α2β1	RGD
	α4	FN, V-CAM 1	EILDV
	α5	LN, FN	RGD
	α6	LN, MR, KN	
	α7	LN	
	α8		
	αv	VN, FN	RGD
β2	αL	I-CAM 1, I-CAM 2, I-CAM 3	
	αM	iC3b, FG, factor X, I-CAM 1	QXRLDS
	αX	FG, iC3b	
β3	αIIb	FG, FN, VN, TSP, vWF	RGD, KQAGDV
	αv	VN, FG, TSP, FN, COL, OP, TN, vWF	RGD
β4	α6	LN, KN	
β5	αv	VN	RGD
β6	αv	FN	RGD
β7	α4	FN, V-CAM 1	EILDV
	αIEL		
β8	αv		RGD

4.3 Structural and functional considerations of the integrin subunits

Both the integrin subunits are large transmembrane molecules, and electron microscope studies reveal a molecule with a globular head and two tails (Carrel *et al.*, 1985). These observations in addition to the subunit primary structure suggests that the two molecules interact at their N-terminal regions to form the globular extracellular domain and are connected to the the C-terminal cytoplasmic portion by a short single pass transmembrane domain. The extracellular and the cytoplasmic domains appear to have distinct, yet interrelated, functions.

4.31 Cytoplasmic domains

The short cytoplasmic domains of both the α and β integrin subunits consist of 20-50 amino acid residues. The β4 subunit is the exception having a cytoplasmic domain of more than 1000 amino acids. The cytoplasmic domains of the β subunits are highly conserved while those of the α subunits are divergent (Marcantio and Hynes, 1988). There is ample evidence indicating that the cytoplasmic domains interact with the cytoskeleton (Burridge *et al.*, 1988; Hayashi *et al.*, 1990; Solowska *et al.*, 1991; Miyamoto *et al.*, 1995), and this activity has been localized to both α (Filardo and Cheresh, 1994; Kassner *et al.*, 1994; Chan *et al.*, 1992) and β subunits (Elices *et al.*, 1991; La Flamme *et al.*, 1992). Alternate exon splicing in the cytoplasmic regions effects differential ligand binding suggesting that the interactions between the subunits at this site partially regulates signalling from the cytoplasm to the exterior (Altruda *et al.*, 1990; Tamura *et al.*, 1990; Tamura *et al.*, 1991; Toin *et al.*, 1989; Brown *et al.*, 1989). A

general trend has emerged that the α subunit cytoplasmic domains act in modulation or modification of the signal that is transmitted by the β subunit (Chan *et al.*, 1992).

The best studied example of the association of the integrins with the cytoskeleton is the assembly of structures known as focal adhesions, which act as sites for actin filament anchorage to the plasma membrane of adherent cells. While focal adhesions are peculiar to cultured cells, they are however analogous to the dense adhesion plaques of smooth muscle, myotendenous junctions, and the sites of adhesion between cells and the basement membrane. The association of the integrin molecule with the actin cytoskeleton in focal adhesions is not direct and requires a number of additional molecules. The use of high affinity peptide analogs of normal ligands reveals that the ability to form the assembled cytoskeleton (aggregation) is a distinct function from ligand binding, and requires either multivalent matrix or activation of the receptor by antibodies (Volz, 1993; Miyamoto *et al.*, 1995). The activation of integrins by antibodies is thought to involve the alteration of conformation of the molecule at extracellular sites that results in a change of the receptor to a high affinity state (Neugbauer and Reichardt, 1991). This change in extracellular conformation mimics a normal process that appears to be regulated through the cytoplasmic portion of the integrin (Miyamoto *et al.*, 1995). The cytoplasmic domains of the β2 subunit contains highly conserved serine residues that are phosphorylated *in vivo*, however phosphorylation at these sites has been dissociated from the binding activity of the molecule and the significance of these events is unknown (Chatila *et al.*, 1989). In cells transformed with the Rous sarcoma virus the β1 and β3 integrins are phosphorylated at a cytoplasmic tyrosine residue, and this is correlated with

the loss of adhesion (reviewed in Hynes, 1992). Despite this evidence it remains unclear if phosphorylation plays a role in regulation of integrin mediated adhesion *in vivo*.

The β4 integrins are localized to hemidesmosomes and the distinctive cytoplasmic domain of this subunit is thought to interact with the intermediate filaments anchored in these structures (Sonnenberg *et al.*, 1991).

4.32 Extracellular domains

The extracellular domains of both α and β subunits contain a large number of cysteine residues that act in the formation of intramolecular disulphide bonds (Calvete *et al.*, 1989, 1991). Despite the conservation of these regions among diverse subunits, deletion mutations suggest that those regions close to the plasma membrane do not play an important functional role in the mature β subunit (Wippler *et al.*, 1994). Characteristic of the β subunit is a four fold repeat of a cysteine rich domain in the extracellular region proximal to the cytoplasmic membrane. The α subunit contains a seven fold repeat, the N-terminal four of which are thought to act in divalent cation binding (D'Souza *et al.*, 1990). The proper presentation of the α subunit on the surface of the cell requires the presence of the β subunit extracellular domain, and the interaction of the two subunits is localized to the cation binding sites on the α subunit (Gulino *et al.*, 1992). Some α subunits contain an N-terminal insertion called the I domain. This insertion has been identified as a site where divalent cation discrimination takes place (Dransfield *et al.*, 1992), as well as a ligand binding site for various factors which activate the β2 receptors (Kamata and Takada, 1994). There are a number of integrins which are known to bind

ligand in a divalent cation independent manner through the α subunit. Some of these contain the I domain described above. Others have an α subunit that is post-transcriptionally cleaved into two molecules, which are subsequently rejoined by a single disulphide bond. The mechanisms by which such a conformation gives independence from divalent cations is unknown. However, all the integrins that are localized to focal adhesions contain the cleaved α subunit. These integrins are thought to bind plasminogen at an extracellular site close to the cell membrane correlating closely with the cleavage site in these subunits (Calvete *et al.*, 1990).

4.33 Divalent cation binding

In general integrins mediate divalent cation dependant adhesive events. It is clear that there are divalent cation sites of differing affinities, a high affinity site being required for subunit association, and three low affinity sites needed for ligand binding (Rivas and Gonzales-Rodriguez, 1991). There is also evidence that the active form of the receptor involves a conformational change to the α subunit similar to that observed when a single cation is displaced upon peptide binding (D'Souza *et al.*, 1994) This situation is further complicated by the observation that the use of non-displacable cations to abolish ligand binding localizes this function to the β subunit (Smith and Cheresh, 1991). This is supported by evidence from patients with Glazmann's thrombosthenia indicating that a point mutation in the β subunit abolishes ligand binding. This mutation results in a conformational change to the receptor similar to that observed when a cation is displaced, and the location of the mutation is at a site that bears a strong resemblance to the Ca^{2+}

binding domain of calmodulin (Loftus *et al.*, 1990). This suggests that the binding site for at least one of the cations may be shared and upon binding ligand or receptor activation this cation is displaced (Loftus *et al.*, 1994).

5.0 Ligand binding by integrins

The integrins bind a diverse array of molecules that include components of the ECM, cell surface molecules of the IgG superfamily, bacterial and viral proteins (Van Nhieu *et al.*, 1991), and serum proteins. The binding sites for these ligands has largely been found to be at a site distal to the cytoplasmic membrane that is dimeric. This dimeric ligand binding site utilizes portions of both the α and β subunit (Wippler *et al.*, 1994; Santoro and Lawing, 1987). The association of α and β subunits does not confer ligand binding specificity as there are integrins of identical subunit composition that bind distinct ligands (Neugbauer and Reichardt, 1991; Kirchhofer *et al.*, 1990), and the same ligand is bound at the same site by receptors displaying diverse subunit composition (Mould *et al.*, 1994).

All the identified integrin ligands that bind at the dimeric site contain a motif with an essential aspartic acid (Figure 1). The primary structure of integrin ligands suggest a highly labile molecular structure, the aspartic acid being presented at the junction of two β turns. This conserved structure may explain the overlapping binding abilities of many integrins, and possibly accounting for the variable affinities that are observed (Smith, 1994). It is thought that the essential aspartic acid residue initially interacts with the

divalent cation that is shared by the α and β subunit and that upon ligand binding the cation is replaced with the aspartic acid residue (Smith and Cheresh, 1991).

The best characterized of the integrin ligand motifs is the arginine-glycine-aspartic acid (RGD) sequence that has been found in a number of ECM proteins (figure 1). The integrins known to bind this motif, α5β1, αvβ3, and αIIbβ3, have divergent functions suggesting that function is mediated elsewhere (Albeda *et al.*, 1990; Phillips *et al.*, 1991). The ability of the receptor to bind this sequence is absolutely dependant upon the aspartic acid residue. Substitution of L-Aspartic acid with D-Aspartic acid results in the abolition of ligand binding (Pierschbacher and Ruoslahti, 1987). As expected by the ability of the integrin to discriminate between stereoisomers of aspartic acid three binding sites have been identified. Two of these localize to the α subunit and a single site has been identified on the β subunit (Smith *et al.*, 1990). The ability of integrins to bind peptides overly simplifies the ligand binding mechanisms of these receptors. Integrins bind a complex matrix with a greater affinity than that displayed for isolated components of the ECM (Morla *et al.*, 1994). It is also evident that sequences peripheral to the RGD site in the ligand participate in ligand-receptor interaction likely providing contextual signals (Bowditch *et al.*, 1991; 1994). Cells plated on RGD containing peptides respond differently than those plated on fibronectin suggesting that ligand structure not only affects receptor affinity but also the signal transmitted by the receptor (Massia and Hubbell, 1991). Thus, studies with peptides have provided us with much of our understanding of the binding characteristics of integrins and indicate contextual interpretation of these motifs within a complex matrix is important.

5.1 Other integrin ligands

A number of other ECM molecules that do not contain the conserved aspartic acid residue are also bound by integrins. Some of these ligands interfere with the binding of ligands that contain the essential aspartic acid but not with peptides derived from those molecules. This observation suggests that the two molecules are bound at overlapping, or adjacent sites. Recent evidence indicates that a second binding site localized to the α subunit is distinct from the dimeric site described earlier (Kamata and Takada, 1994). This ligand binding site on the α subunit appears to be cation independent (the I domain and the extracellular sequence proximal to the cellular membrane on the cleaved subunits that binds plasminogen) and is independent of the β subunit (Michishita *et al.*, 1993). This indicates that integrins have more than one site at which they interact with ligands. Despite these observations, presentation of the receptor on the cell surface is dependant upon the association of the α subunit with the β subunit (Wilcox *et al.*, 1994). Thus, the functional implications of ligand binding to the α subunit cannot be distinguished from that requiring both subunits.

There has also been some recent evidence that α3β1 integrins may function in intercellular contact through homophilic binding (Sriramarao *et al.*, 1993). As well as an implied association of the α2β1 and α3β1 in cell-cell contacts (Symington *et al.*, 1993). A cell-cell adhesion role for integrins is indicated by the observation that the antibody BV7, which is directed against the β1 subunit, blocks α2β1 mediated adhesion of colon carcinoma cells to endothelial cells. The cell-cell adhesion mediated by α2β1 is divalent

cation independent and is not blocked by inhibitory antibodies to selectins, or the VCAM and ICAM ligands of the α2β1 integrin. The α2β1 integrin has been reported to bind the α3β1 integrin (Symington *et al.*, 1993), however the α3β1 integrin is not found on the surface of endothelial cells. BV7 also interferes with cell adhesion to laminin, collagen, and fibronectin (Martin-Padura *et al.*, 1994) suggesting there are adhesive events mediated by integrins that we are unaware of.

A number of organisms use integrins as mediators for the invasion of tissues or cells. Viruses, bacteria, and *Leishmania* all have molecules that contain or mimic the RGD site found in ECM components, and use integrins as a site of initial adhesion in the process of internalization (Smith, 1994). Snake venom contains a family of molecules termed disintegrins, which interferes with the binding of the integrin αIIbβ3 to fibrinogen preventing clot formation. Disintegrin-like sequences have now been found in other vertebrates and are thought to act as normal ligands for integrins, although a definitive role for these molecules remains unclear (Blobel *et al.*, 1992).

5.2 Modulation of ligand-integrin affinity

The binding of an integrin to its ligand appears to be a complex multi-step process. The use of integrins by migrating cells indicates that there must be a dynamic modulation of adhesion. The analysis of cells under various laminar flow conditions has shown that transient adhesive events can stem from the low ligand affinity of certain integrins, several of which have been implicated in cell migration (Tozeren *et al.*, 1991). Interestingly, this low affinity adhesion may be regulated by the discrimination between

divalent cations (Smith, 1994) and the affinity for distinct ligands by the same integrin (Mould *et al.*, 1994; Makarem *et al.*, 1994).

An important feature of the integrin-ligand interaction is that the spacing of the ECM motifs bound by integrins appears to be vital to the affinity of the interaction. Evidence suggests that the spacing of the ECM motifs must be close enough that integrin receptors cluster, although clustering appears to be regulated from within the cell and can be dissociated from receptor occupancy (Massia and Hubbel, 1991). This clustering is a recurrent theme in signal transduction by integrins and is likely essential since integrin binding of soluble ligands often does not result in receptor clustering, cytoskeletal assembly, or signalling events (Miyamoto *et al.*, 1995).

Integrins appear to be able to aid in the assembly of fibronectin and collagen matrices (Fogerty *et al.*, 1990; Bette *et al.*, 1994). The sites bound by integrins on these molecules during matrix assembly are distinct from those bound during cell adhesion and migration events, and in general are of lower affinity (Bette *et al.*, 1994). There is evidence that various ECM components have the ability to self assemble and integrins may act in establishing localized concentrations of matrix components as opposed to actively assembling the ECM (Morla *et al.*, 1994). Some of the integrin ligands such as tenascin, laminin, and thrombospondin appear to have anti-adhesive activity when they are presented in a soluble form and this may represent a general mechanism for regulation of adhesion (Adams and Watt, 1993).

Thus integrins are primarily receptors that mediate adhesion. It is clear that they are involved in a number of processes and that these can often be localized to distinct

domains on the receptor. Some of these processes have a role *in vivo*, while others, such as divalent cation independent ligand binding may be a consequence of experimental conditions.

6.0 Signal transduction by integrins

Integrins provide continuity between the extracellular environment and the cytoplasm, suggesting that they may play a role in transmitting signals across the cellular membrane. Until recently, the only cytoplasmic association that integrins were known to have were with the actin cytoskeleton. Many of the effects that were attributed to integrin function were thought to stem from this association. Adhesion regulates growth, gene expression, and organization of the cytoskeleton, and it is now becoming clear that these signalling events likely stem from cooperative interactions between a number of classes of receptors (O'Brien, 1995). It is important to emphasize that these signalling events still require integrin interaction with the ECM and it remains unclear how the signalling cascade is initiated. The following discussion is concerned more with the effects of integrin mediated signalling rather than the processes that initiate these signals.

6.1 Integrin mediated phosphorylation

Ligand binding by the αIIbβ3 platelet integrin results in the phosphorylation of a number of cytoplasmic proteins. In platelets that lack the αIIbβ3 integrin the same cytoplasmic proteins are not phosphorylated indicating that the occupancy of this receptor is essential to these events (Clark and Brugge, 1995). In fibroblasts, antibodies directed

against α or β subunits induce phosphorylation of a component of the focal adhesion complex known as pp125FAK (focal adhesion kinase). Phosphorylation of pp125FAK is not a direct consequence of ligand binding as it can be dissociated from receptor occupation through the use of non-activating peptides (Miyamoto *et al.*, 1995). Integrin binding of peptides or ECM fragments does not initiate phosphorylation of pp125FAK, while binding of a complex matrix or antibody mediated receptor clustering does suggesting that other integrin mediated signalling processes are needed (Guan *et al.*, 1991). Phosphorylation activates pp125FAK and results in a cascade of phosphorylation activity (Kornberg *et al.*, 1991; Kanner *et al.*, 1990). Recent evidence suggests that the activation of pp125FAK by phosphorylation can lead to the initiation of gene expression through translocation of MAP kinase resulting in transcription factor phosphorylation and relocation to the nucleus (Chen *et al.*, 1994). Other substrates of pp125FAK include the proteins paxillin and tensin (Burridge *et al.*, 1992) both involved in the assembly of the actin cytoskeleton in focal adhesions, a characteristic of integrin mediated adhesive events. The αvβ3 integrin is associated with a different cytoplasmic protein that is phosphorylated upon exposure of adherent cells to platelet derived growth factor (PDGF). The phosphorylation of this protein results in the inactivation of the receptor and consequently the loss of adhesion (see below, Bartfield *et al.*, 1993). Primary sequence of cloned integrin subunits, and the binding of non-activating peptides indicates that integrins have no kinase activity themselves.

6.2 Growth factors and integrins

The ability of cells to respond to many growth factors is dependant upon cell adhesion to the ECM (Schubert and Kimura, 1991). The role that integrins play in growth factor mediated signalling is unclear, however, exposure to growth factors can initiate upregulation of integrin expression in adherent cells. Increased transcription, synthesis, and processing of integrin subunits has been observed in a number of cell types upon treatment with epidermal growth factor (EGF) or transforming growth factor β (TGF-β). Elevated integrin expression is correlated with increased migration or spreading of cells on collagen matrices (Fujii *et al.*, 1995; Bellas *et al.*, 1991; Wahl *et al.*, 1993). The expression of novel integrins on the surface of cells exposed to growth factors does not need to involve *de novo* transcription, as both the αv and β1 subunits appear to be recruited from post-translational sources (Sheppard *et al.*, 1992). Recent evidence suggests that the αvβ3 integrin in particular is involved in growth factor signalling through receptor mediated phosphorylation of a high molecular weight protein associated with the β3 subunit (Bartfield *et al.*, 1993; Vuori and Ruoslahti, 1994). The induction of the cascade that leads to phosphorylation of this complex is absolutely dependant upon cell adhesion to vitronectin. I the case of exposure to PDGF, phosphorylation results in the disruption of the receptor/ligand complex. Culturing of cells in the presence of growth factors can induce events closely associated with integrin function such as the synthesis of ECM components (Loeser, 1990), and proteinases directed at the ECM (Wahl *et al.*, 1993).

6.3 Other intracellular events mediated by integrins

Integrins are also known to mediate a number of other intracellular events such as calcium transients, pH changes and activation of second messenger pathways (Schwartz, 1994). The most pertinent of these to this study is perhaps the role that calcium plays in integrin signalling events. Assays of integrin function suggested that these molecules may act as calcium membrane channels (Richter, 1990). This interpretation was likely due to contamination of the integrin preparation with a calcium channel that is intimately associated with the receptor and activated when the integrin binds ligand (Ryback and Renzulli, 1989). Although the role that the association between the integrin receptor and the calcium channel plays *in vivo* remains unclear, it is evident that cells exhibit calcium transients while migrating on a variety of ECM substrates (Jaconi, 1991). Disrupting cell attachment to these substrates with antibodies directed against the αmβ2 integrin blocks the calcium transients and interferes with migration. Similar results are obtained with cells migrating on vitronectin using buffers that inhibit calcium influx. Thus, it appears that modulation of intracellular calcium levels are tied to the mediation of integrin function, although there is no direct evidence for this *in vivo*.

Perhaps the best example of integrin regulation of gene expression is in mouse mammary epithelial cells where occupancy of β1 integrins results in the synthesis of β-casein in the absence of any tissue morphology or signalling from growth factors (see later discussion, Streuli *et al.*, 1991). Antibodies against the β1 subunit induce the transcriptional expression of proteinases (Werb *et al.*, 1989). Fragments of ECM molecules or peptides also induce proteinase synthesis while intact ECM does not, again

suggesting that a complex matrix is required for proper integrin signalling (Werb *et al.*, 1989). Although cells exhibit a number of integrins on their surface only a subset of these act to induce gene expression. To date only integrins that contain the β1 subunit are known to act in regulating gene expression (Yurochko *et al.*, 1992).

The role that integrins play in intracellular signalling events is not clear due to our inability to discriminate between convergent intracellular pathways. It is clear that the association of integrins with their ligands is essential for the initiation of many signalling cascades. This signalling results in cell movement, adhesion, or gene expression. It is these events that are likely at play during the developmental processes mediated by integrins.

7.0 The role of integrins in development

Due to the importance of the cellular movements that are observed in early development the integrins are of considerable interest. As previously discussed, the role that these cell rearrangements play in signalling, also suggests that molecules such as the integrins may be integral to mechanisms that act in development. Most of the processes that are mediated by integrins are highly correlated with pattern formation, determination, or differentiation events. A common theme to all these situations is the tissue specific and temporal regulation of integrin subunit expression. While there are a large number of developmental events suspected to be mediated by integrins only a few of the better studied examples using various model systems are discussed below (see Lallier *et al.*, 1994 for a review).

7.1 The role of integrins in *Xenopus* development

Xenopus embryos provide a manipulatable system for the examination of signalling events that occur during gastrulation and pattern formation. The elucidation of the mesoderm and neural induction signalling pathway (reviewed by Kessler and Melton, 1994) has made this a good model system for examination of the developmental regulation of integrin function. A number of β integrin (Ransom *et al.*, 1993) and α integrin (Whittaker and DeSimone, 1993) subunits have been cloned and their patterns of expression determined. As expected by its promiscuous subunit composition and ligand association, the β1 subunit has a ubiquitous pattern of expression from fertilization through to neurulation (Ransom *et al.*, 1993). The β1 subunit is expressed on the surface of the oocyte, internalized upon fertilization, and reappears on newly synthesized cellular membrane during cleavage although no function is attributable to the molecule at this time (Gawantka *et al.*, 1992). The β3 subunit has been localized to the bottle cells at the base of the neural groove, and in later development is found in the blood islands (Ransom *et al.*, 1993). No information is available on the distribution of the other subunits in *Xenopus* embryos at this time.

While little information is available concerning the distribution of the β subunits, the α subunits have been localized to a number of sites that are active in morphogenesis. The α3 subunit is present at the dorsal lip of the blastopore during involution of the mesoderm, and at later stages is found in the notochord (Whittaker and DeSimone, 1993). The localization of the α3 subunit to the notochord is of particular interest as it is

known that the β1 subunit, which normally pairs with α3, and fibronectin are not involved (Smith *et al.*, 1990). It is also possible that the α3 subunit binds the fibronectin matrix that overlies the blastocoel (Keller and Jansa, 1992) acting to guide notochordal mesoderm migration during early gastrulation. Three α subunits appear to define anterior-posterior regions of the neural plate. The anterior portion of the neural plate and possibly some of the cranial neural crest express the α5 subunit. The middle portion of the neural plate is defined by the α6 integrin. Later in development the α3 subunit decreases in expression in the notochord and is found in the forebrain, while the α5 and α6 subunits down regulate in neural ectoderm and are subsequently found in mesoderm (Whittaker and DeSimone, 1993). The α5 and α6 subunits are expressed during the differentiation of the neural plate and differential adhesive events may be responsible for segregation of this tissue from the underlying mesoderm (Lallier *et al.*, 1994).

The surface localization of individual integrin molecules does not always correlate with the presentation of functional receptors, and it is known that various integrins require activation before becoming competent to bind ligand. There is evidence for integrin function in a manner that correlates well with the spatial, temporal, and tissue specific localization of the integrin subunits described above. The culture of animal caps isolated from *Xenopus* blastula results in the formation of ectodermal tissues. Incubation of these explants in the presence of Activin (a TGF-β homologue) results in the formation of notochordal tissues that undergo shape changes reminiscent of those occurring during notochord elongation. Cells isolated from these mesodermal tissues can attach and spread on fibronectin matrices in an RGD dependant manner, while those cells not

exposed to activin do not, indicating integrin mediated adhesion is activated by TGF-β (Smith *et al.*, 1990; Lallier *et al.*, 1994). While the α5β1 receptor is the most common fibronectin receptor, attachment of activin treated animal cap cells to fibronectin induces transcription of α subunits other than α5 (Whittaker and DeSimone, 1993). It is likely that signals stemming from the occupation of the FGF receptor by activin are activating receptors already present on the surface of the cells. Such a mechanism would be consistent with the organizing centre hypothesis, which suggests that gradients of growth factors are responsible for defining patterns in early *Xenopus* embryos. As discussed earlier, these patterns are established during times of cell rearrangements and these experiments suggest that integrins play a role in these events.

The indication that integrins are playing a role in, or are a result of cytokine-mediated signalling events that define tissues, suggests that some of the intracellular events mediated by integrins should be detectable in these tissues. Phosphorylation of pp125FAK can be detected in *Xenopus* embryos and this activity is concentrated around the involuting mesodermal cells, correlating with the spatial and temporal expression of the α3 integrin (Lallier *et al.*, 1994). While it is difficult to form a cohesive story from the available information, there is evidence in *Xenopus* for integrin-mediated events that dictate the acquisition of tissue identity. The acquisition of tissue identity also seems to be regulated through signalling pathways that are known to activate integrin function. This suggests that progressive determinative events are active in regulating functional changes in integrin expression. There is also evidence for integrin-mediated intracellular events,

including the initiation of gene expression, that are characteristic of those observed *in vitro.*

7.2 Integrin functions in chick development

A number of insights into integrin function have been provided by studies of neural crest cell migration in chick embryos. Central to these studies is the well documented localization of ECM molecules (Perris *et al.*, 1993; Newgreen and Thiery, 1980; Tucker and Erickson, 1984) and this has been correlated with the use of integrins by neural crest cells *in vivo* and *in vitro.* An examination of the αv subunit and its association with vitronectin in the embryo suggested that there are three αv associated integrins that mediate distinct functions (Delannet *et al.*, 1994). Using *in vitro* assays two of these, the αvβ3 and αvβ5, receptors were found to mediate migratory activity, whereas the αvβ1 receptor was found to function in static, high affinity, cell adhesion. The synthesis and localization of vitronectin to the surface of the cells expressing these integrins *in vivo* suggests that perhaps one of the receptors (αvβ1) is used to assist in matrix assembly, while the other receptors function in migratory activity utilizing the assembled matrix. Further complicating the issue is the observation that experiments using blocking antibodies, indicate that there are at least three other integrins that are acting in the adhesion or migration on vitronectin (Delannet *et al.*, 1994).

The complexity of integrin expression on the surface of neural crest cells has also been observed in immunoprecipitates using an antibody directed against the β1 subunit, which coprecipitates a number of α subunits (Muschler and Horwitz, 1991). Such

receptor diversity is expected due to the complex nature of the ECM encountered by the migrating cells (Erickson and Perris, 1993). There is evidence that the spatial localization of specific ECM components results in the alteration of receptor usage and as a consequence a change in cellular behavior. An example of this is the different roles played by the the β1 integrins in the cranial and trunk neural crest cell populations during migration (Erickson and Perris, 1993). Similarly, the use by neural crest cells of the myotome basal lamina in preference over the sclerotome basal lamina, results in a change in the direction of migration and the acquisition of specific fates, although this activity has not been attributed specifically to integrins (Fosney *et al.*, 1994). Alternatively, melanocytes appear to regulate the use of migratory pathways through cell surface molecules that become functional as the cells differentiate (Erickson and Goins, 1995). This suggests that directional migration in these cells is a result of acquisition of the ability to utilize pathways rather than the pathways determining fate. There is some evidence that the adhesive affinities of integrins for various ligands may also result in the localization of neural crest cells at target sites (Lallier *et al.*, 1992).

Whereas there is evidence for integrin function in neural crest cell migration, little is known of the identities of the α subunits that act in these movements. The use of anti-sense oligonucleotides to inhibit the attachment of neural crest cells to ECM molecules *in vitro* has revealed a potential role for α1 subunits while suggesting that α5, α6, α7 have no role in ECM mediated cell migration (Lallier and Bonner-Fraser, 1993), although Muschler and Horwitz (1991) provide evidence for α5 expression on migrating neural crest cells. While there is ample evidence *in vitro* for integrin function in neural crest cell

migration and the correlation between ECM component localization and neural crest cell behavior *in vivo* is tantalizing, however few integrins have been identified and the roles that they may play remains elusive.

7.3 Integrin functions in mouse development

The mouse has become a powerful model for developmental studies due to the ability to create inheritable null mutations in specific genes. This system has been used to examine the role that the α5 subunit plays in early development (Yang *et al.*, 1993). The formation of mesoderm in α5 deficient embryos is not inhibited, but the morphological disruption of mesoderm is so great that the mutation is lethal. Cells isolated from these embryos can attach and spread on fibronectin, as well as act in the assembly of the matrix. These results indicate that mesoderm formation and the movements of gastrulation can be dissociated, and that both these events can be partially compensated for by other adhesive receptors that utilize fibronectin as a ligand. In a reciprocal experiment George *et al.*, (1993) have created mice with a null mutation for fibronectin that display an earlier embryonic lethal phenotype. There appears to be a general lack of organization of the notochordal mesoderm resulting in the embryonic anterior/posterior axis being severely shortened. These results seem to correlate well with those observed in *Xenopus* (Smith *et al.*, 1990) although it appears that these events may be mediated by a different subset of integrins. An interesting aspect of these experiments is that the α5β1 integrin binds the RGD sequence in fibronectin along with five other integrins (Figure 1), thus the signalling

events stemming from a single ECM binding motif appear to be nonredundant as the α5 mutation is lethal.

Yang *et al.* (1995) have also recently produced knockout mice for the α4 integrin subunit and in these animals the placenta and epimyocardium do not differentiate properly. Developmental failures are observed in the apposition between two differentiating structures suggesting that the integrin acts in defining or establishing tissue boundaries. This is confirmed by a reciprocal experiment in which the α4β1 ligand, VCAM-1, was effectively removed using homologous recombination (Kwee *et al.*, 1995). In α4 deficient mice the epimyocardium and the placenta also fail to develop properly. Immunohistological localization of the α4β1 and VCAM-1 confirm that they are expressed on opposing cell surfaces. In this situation the same defects are observed in both receptor and ligand mutations suggesting that there is no redundancy in the role that α4β1 and VCAM-1 play in the embryo.

The mouse has provided one of the clearest examples of how the ECM regulates gene expression. Mouse mammary epithelial cells (MMEs) have been shown to be dependant upon the ECM of the basement membrane for their ability to produce the milk protein β-casein. MMEs can be grown in a three dimensional matrix of laminin producing unpolarized single cell cultures that produce β-casein. This result is dependant upon the composition of the ECM as cultures in collagen matrices do not have the ability to initiate transcription of the β-casein gene until they synthesize their own laminin based matrices. Addition of an anti-β1 integrin antibody to cells cultured on laminin eliminates β-casein transcription, indicating the induction of β-casein transcription is a direct result of integrin

binding (Streuli *et al.*, 1991). These results correlate well with the situation *in vivo* during mammary gland differentiation and regression. The regression of the mammary gland has been attributed to the balance between laminin proteinases and proteinase inhibitors (Talhouk *et al.*, 1992). During periods of high inhibitor expression the basement membrane is intact and β-casein transcription is high. However, as the inhibitor levels decrease and the proteinase becomes active the basement membrane becomes degraded and the the levels of the β-casein transcript decrease. The MMEs eventually detach from the degraded basement membrane and undergo apoptosis in the absence of cell adhesion. Neutralization of the proteinase by addition of exogenous inhibitor, results in the failure of basement membrane degradation, the MMEs do not undergo apoptosis and continue expression of β-casein (Strange *et al.*, 1992). There is a possibility that the transcriptional regulation of β-casein *in vivo* is regulated by factors other than integrin binding, however, the correlative evidence that the ECM regulates tissue specific gene expression *in vitro* is compelling.

7.4 *Drosophila* development and integrins

There have been two α and two β subunits identified in *Drosophila*. One of the β subunits (βv) is expressed in highly restricted spatial and temporal manner in the midgut (Yee and Hynes, 1993). βv likely forms association with the PS2 α subunit (Brabanet and Brower, 1993) and is thought to mediate the morphogenetic movements that form the gut (Yee and Hynes, 1993). The other integrin subunits have been localized to wing formation as well as the attachment of body wall muscles (Leptin *et al.*, 1989). The

situation in the wing is the best characterized. The *Drosophila* PS1 and PS2 subunits are associated with the common PS3 β subunit. In the wing the PS1α subunit is localized to the dorsal surface, and PS2 α subunit is found on the ventral surface. Mutations that abolish the expression of either of these integrin subunits results in epithelial delamination in the wing (Brabanet and Brower, 1993). Initially it was thought that the PS1 and PS2 containing integrins bound each other, since no ECM components have been found between the opposed epithelia. However, null mutations in the common β subunit do not mimic those of the α subunits, and it is unlikely the receptors bind each other (Brown, 1994). The regulation of the transcriptional pattern of the αPS1 subunit in the ventral wing rudiment is negatively controlled by the apterous protein, a homeodomain containing protein known to regulate the development of pattern during *Drosophila* embryogenesis (Blair *et al.*, 1994). It appears that the cascade of gene expression that establishes pattern in the *Drosophila* embryo directly regulates the expression of cell adhesion molecules which act in the movements of morphogenesis that follow pattern formation. While little is known of the *Drosophila* integrin ligands, a novel ECM cDNA, tiggrin, has been isolated and the expressed protein supports cell attachment and spreading in an RGD dependant manner that is mediated through the αPS1βPS integrin (Fogerty *et al.*, 1994). The common theme concerning integrin expression in *Drosophila* is that expression is restricted to certain tissues and appears to define boundaries, either between functional domains of an organ system, or through regulation by the pattern formation genes expressed early in development.

Presently the evidence for a functional role for integrins during development is strong. There are a number of conclusions that arise out of what we presently know. It is clear that there are domains of spatially restricted expression for various integrin subunits suggestive that these molecules play distinct roles. The observation that these patterns of expression are modified temporally in a fashion that crosses tissue boundaries indicates that the roles played are conserved in diverse tissues. The expression of integrins is also closely correlated with the movement of cells or the establishment of tissue boundaries that define cellular identity indicative of a role in determination events. It is evident that in the model systems described above that the complexity of the embryos restricts the ability to interpret observations. The complexity of the embryos used as developmental models is often overlooked in favor of other factors. For instance the mouse offers the possibilities of directed inheritable null mutations, *Xenopus* has proved to be a fruitful model system for the elucidation of signalling pathways through dominant negative interference of receptor function, while *Drosophila* and *C. elegans* have the advantages of genetic manipulation. As integrins are molecules that mediate the adhesiveness of cells it would perhaps be better to use a model organism that exhibits a simple morphology, yet is complex enough to allow correlation of cell movements to other model systems. The sea urchin embryo is an attractive system for experimental embryology due to the simple structure, clarity of the embryo, well characterized embryology, and ease of manipulation both in terms of cells as well as at a molecular level. Importantly, the cellular behavior during morphogenesis is invariant and due to the limited number of cells is straightforward enough to allow for lineage analysis. Despite

this the feeding pluteus stage of the sea urchin *Strongylocentrotus purpuratus* is significantly complex enough to contain specialized derivatives of all three primary germ layers. The described attributes make the sea urchin embryo an attractive model system for the analysis of specific adhesion receptors.

8.0 Review of sea urchin morphogenesis.

The early life cycle of *Strongylocentrotus purpuratus* involves the elaboration of a free swimming feeding larva from the fertilized egg. The planktotrophic larvae eventually settles and metamorphoses into the adult form. While the events associated with metamorphosis are complex, the morphogenetic events associated with the formation of the feeding pluteus larvae are simple and provide an elegant model system for cell movements during early development. The following description is concerned only with the developmental events that result in the formation of the pluteus larvae and as such address only the early events that preceed the feeding stage (Figure 2).

The gametes are spawned and fertilization is external. Cleavage is initiated upon fertilization with the first cleavage being completed within 150 minutes (at 15° C) postfertilization (PF). The subsequent 10 cleavage cycles are nearly synchronous and occur every 45 minutes. The fourth cleavage is unequal and produces the micromere lineage localized at the vegetal pole of the embryo. The micromere lineage provides inductive cues that establish an animal/vegetal polarity to the embryo and act in the determinative events that establish the primary germ layers. The ciliated blastula consists of a hollow sphere bounded by a simple epithelium consisting of equal sized blastomeres,

Figure 2: Representitive stages of early sea urchin development.

A. Fertilized egg. Arrow indicates the fertilization envelope.

B. Two cell embryo. Arrow indicates the hyaline layer.

C. 16 cell embryo. The micromeres are visible at the vegetal pole (arrow). This stage corresponds to what is described in the text as cleavage stage embryos.

D. Primary mesenchyme blastula. The embryo has hatched and is free swimming. Ingressing cells (arrow) visible in blastocoel are primary mesenchyme cells (PMCs). This stage corresponds to what is described as blastula in the text.

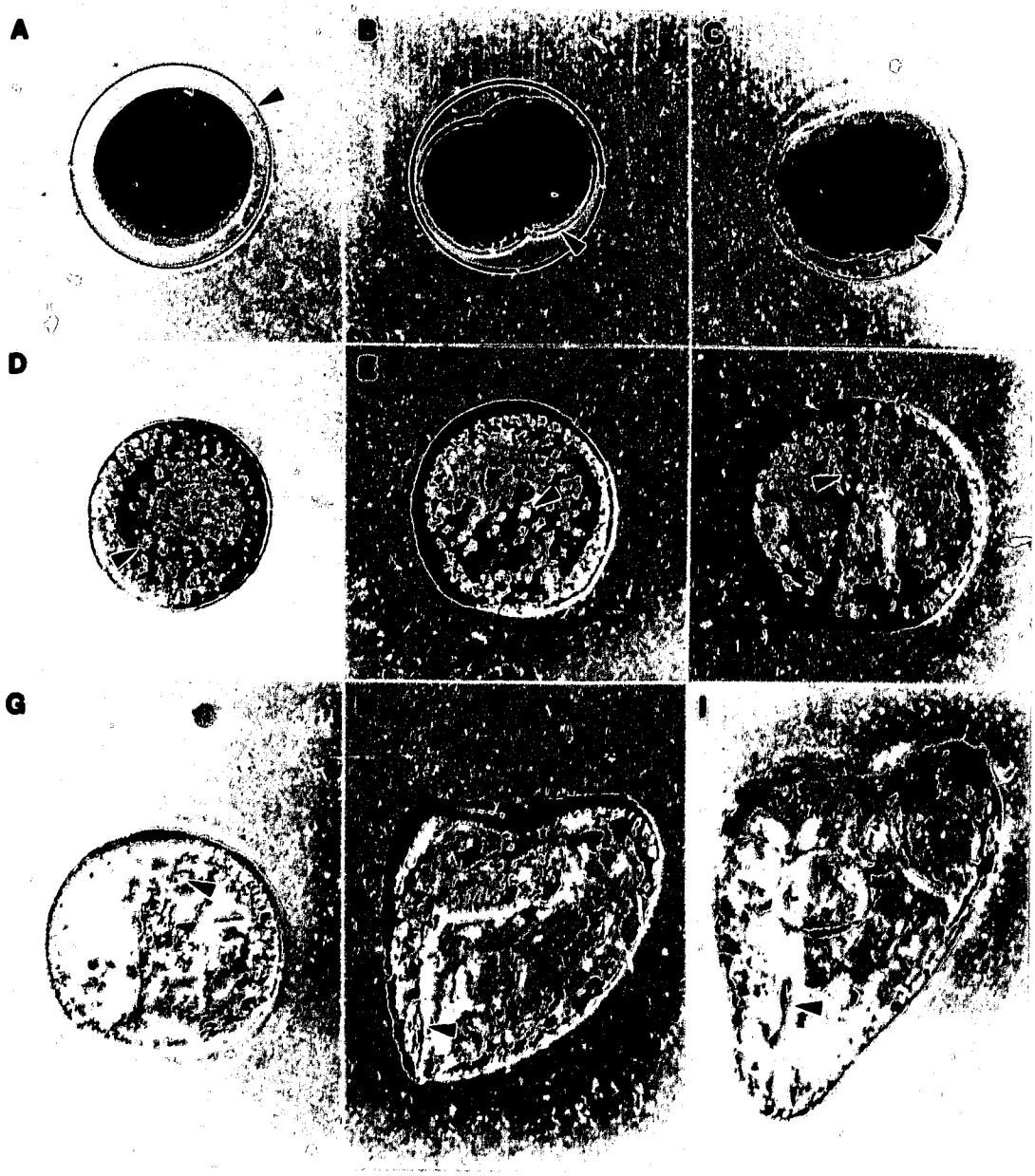
E. Early-gastrula. The floor of the embryo has invaginated to form the archenteron. The PMCs lie lateral to the archenteron, and pigment cells are detaching from the tip of the archenteron (arrow).

F. Mid-gastrula. The secondary mesenchyme cells (SMCs) at the tip of the archenteron send out filopodia (arrow) that contact the ectodermal basal lamina. This stage corresponds to that described as gastrula in the text.

G. Late-Gastrula. The SMCs are migrating into the blastocoel (arrow) as the archenteron nears the animal pole.

H. Prism, lateral view. The embryo is taking on the characteristic prism shape (oral surface at the top of the figure). Skeletal rods are visible (arrow). This stage corresponds to that described as prism in the text.

I. Early Pluteus. The embryo is feeding and the digestive tract has matured into three segments, the esophogous, the stomach and the gut. The arrow indicates the skeletal rods.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and hatches at 24 hours PF to become free swimming. At 28-32 hours PF a thickened plate forms at the vegetal pole and a number of cells known as the primary mesenchyme cells, (PMCs) detach and ingress into the blastocoel, eventually taking up positions that form a ring around the vegetal pole of the embryo. Gastrulation is initiated, at 38 hours PF, with the invagination of the vegetal plate to form the archenteron. This is followed by the extension of the archenteron towards the animal pole. During the early phase of gastrulation a population of cells (pigment cells) release from the archenteron. Once the archenteron has extended approximately one third of the distance across the blastocoel, another population of cells termed secondary mesenchyme cells (SMCs), send out long filopodial extensions that form contacts with the ectodermal basal lamina. As the archenteron continues to extend, these cells begin to detach from the tip of the archenteron and migrate into the blastocoel. By 48 hours, the archenteron has fused with the ectoderm and the stomadeum will form at this location. During this time the PMCs form ventral-lateral syncytial clusters that secrete calcium carbonate spicules, eventually forming the skeleton of the pluteus larvae. As the skeleton is elaborated, the larvae begins to assume the shape of a prism. The four sided base forms the oral surface, the lateral sides the aboral surface and the apex defines the posterior end of the embryo. The archenteron becomes segmented by muscular sphincters forming an esophogus, stomach and gut. Four arm buds appear at the corners of the oral surface and are extended as the larvae starts feeding. Phases of development occurring after the early pluteus are not considered here. For the rest of this discussion the stages of development preceeding

gastrulation are referred to collectively as the early stages of development, while those that occur after gastrulation is initiated are referred to as late stages of development.

8.1 Spatial regulated patterning during cleavage

The generation of form in the sea urchin embryo can be ascribed to mechanisms that are both cell autonomous and those that require conditional specification. The autonomous nature of pattern formation originates in presumptive maternal factors that are localized to the vegetal pole of the egg and become constrained to the micromere lineage at the fourth cleavage (Davidson, 1989). While the micromeres are autonomous at this time and slated to form the skeletal rudiments, they are also the major inductive force that drives the establishment of cell lineages along the animal-vegetal pole axis and transplantation of these cells results in the ectopic realization of a secondary axis (Ransick and Davidson, 1993; Hörstadius, 1939). The animal-vegetal axis of the egg is overlain by the bilateral axis of symmetry that becomes discernable in the multicellular embryo by the sixth cleavage (McCain and McClay, 1994) and finally results in the establishment of the primitive embryonic pattern consisting of five territories; the oral ectoderm, the aboral ectoderm, the vegetal plate, the skeletogenic mesenchyme, and the small micromeres (reviewed in Davidson, 1989). Normally the sea urchin embryo displays an invariant cell lineage, however, cell fate is easily disrupted by surgical techniques (Hörstadius, 1939) and embryo manipulations (Hardin, 1989; Logan and McClay, 1994). Treatment of the preblastula embryo with detergents results in a decrease in blastomere adherence resulting in the alteration of cleavage planes and hence cell fate. This suggests that cell

adhesion is responsible for the establishment of the canonical lineages that result from the inductive influence of the autonomous micromere lineage (Langelan and Whiteley, 1985). Thus, at the earliest stages where there are no cell movements, the autonomously established lineage regulates pattern in a manner that is dependant upon static adhesive events.

In the early cleavage stage embryo, the establishment of pattern that demarks four of the five primitive territories, (the micromere lineage being the exception), is delineated by cell boundaries and not by general territories that cross cellular domains (Davidson, 1989). This suggests that the establishment of these patterns is regulated through cell-cell interactions and not by axial specification or localized centres of influence. While these cell fates are established by inductive influences, it is unlikely to be the result of diffusible soluble factors as recombination experiments utilizing various tiers of cells from early embryos clearly establish that these fates are restricted by direct contact (Hörstadius, 1939; Davidson, 1989). Experiments in which LiCl is used to elicit the formation of skeletogenic rudiments in isolated animal caps suggests that this restriction in fate does not stem from maternal sources and that again cell-cell interactions must regulate cell fate (Livingston and Wilt, 1989). This situation is not unique to the animal-vegetal axis, as separation of the four cell stage embryo results in the respecification of the oral-aboral axis that is normally established by the second cleavage (Cameron, 1989; Hörstadius, 1939; reviewed by Davidson, 1989). Therefore, cell adhesion and not cleavage mediated isolation of maternal factors regulates the negative interactions that limit the developmental potential of blastomeres.

8.2 Cell adhesion is essential for mesodermal fates

During cleavage the five primitive territories are established and define tissues that will be elaborated during later development to produce a diversity of cell fates. The regulative abilities of the sea urchin embryo demonstrates that a number of cell fates can be closely correlated with the consequences of adhesive events. The PMCs are derived from the micromere cell lineage and will form the skeletal rudiments of the embryo, even in the absence of all other tissues (Okazaki, 1975). However, these cells are not the only cells with the potential to form skeletal rudiments as LiCl exposure can convert many other cells, with other potential fates, to become skeletogenic. The conversion of skeletogenic cell fate has also been shown to be regulated *in vivo* by the negative interaction of the micromere descendants with neighboring cells. For instance the removal of the PMCs from blastula results in the conversion of SMCs to skeletogenic fates (Ettensohn and McClay, 1988). This conversion of cell fate has been closely correlated with migration patterns of the two populations of mesenchymal cells. If labelled PMCs and SMCs are injected into the blastocoel, these cells will take up their expected positions in the embryo, suggesting that this behavior is mediated by unique cell surface receptors present on the two populations of cells. If the PMCs are deleted, then after a delay a population of SMCs respond by taking up the characteristic positions of the PMCs and express *de novo* molecular markers characteristic of the PMCs. These cells eventually form an anatomically correct skeleton (Ettensohn, 1992). This conversion of skeletogenic fates is normally inhibited by interaction of PMCs with the

SMCs (Ettensohn and Ruffins, 1993). The timing of the ability of the PMCs to arrest the conversion of the SMCs correlates with the localization of the PMCs in clusters that form in the vegetal-lateral regions of the embryo (Ettensohn, 1992). This is strictly a temporal regulation, as PMCs can suppress the conversion event even if their normal localization is disrupted. While the evidence is inconclusive, it would appear at this time that direct contact between the mesenchymal populations mediated by filopodia are responsible for the restriction in SMC fate (Ettensohn and Ruffins, 1993). Thus, it appears that ability of cells to migrate to locations that specify fate is directed by molecules that are expressed on the surface of these cells. Once these cells acquire a spatial identity then they can act through cell surface molecules to influence their neighbors in a temporally restricted fashion.

There is no clear explanation of what regulates the precise positioning of mesenchymal cells within the blastocoel of the sea urchin embryo. There is evidence for the unequal distribution of ECM components within the ectodermal basal lamina. This appears to be due to localized synthesis of these molecules during morphogenetic movements (Chen *et al.*, 1994). PMCs are known to secrete a nonfibrillar collagen at sites of cell attachment *in vitro*, no specific role for this molecule has been found *in vivo* (Wessel *et al.*, 1991). Precise localization of mesenchymal cell populations is also correlated with the expression of population specific cell surface markers (Leaf *et al.*, 1987) suggesting that these may regulate the spatial regulation of these cells. Transplantation experiments in which PMCs from one species acquire the spatial and temporal patterning of the host species indicate that spatial patterning is regulated by the

environment PMCs are found in. However, the spicule structure resembles the donor species (Armstrong and McClay, 1994). This positional pattern probably stems from the ectodermal basal lamina as treatment with nickel disrupts the spicule patterning normally localized to the ventral hemisphere of the embryo. Coincident with the disruption of spicule patterning is the expression of ventral markers in the dorsal ectoderm. A similar situation exists in animal-vegetal half embryos that are supplied with PMCs and examined for their ability to direct spiculogenesis. In this situation animal halves provide cues that define oral spicule features, whereas vegetal halves define aboral features (McClay *et al.*, 1992). PMCs that contact isolated ECM migrate in a highly directed manner suggesting that the ECM provides cues that are dependant upon adhesion but are not adhesive in nature (Solursh and Lane, 1988). These experiments make it clear that the basal lamina of the ectoderm provides environmental cues that direct the temporal and spatial developmental program of the PMCs. While the ectodermal basal lamina plays a dominant role in localization events, the blastocoelar fibrillar matrix appears to be an extension of the ectodermal basal lamina (Kawabe *et al.*, 1981; Spiegel *et al.*, 1989), and it is likely that the PMCs and SMCs use this network for migration. In support of this, injection of antibodies directed against the fibrillar contents of the blastocoel stops mesenchymal cell migrations (Burke and Tamboline, 1990). The highly orientated arrangement of the blastocoelar matrix suggests that it may also act to guide the invaginating archenteron (Kawabe *et al.*, 1981). Therefore it would appear that this complex matrix is capable of directing a number of morphogenetic processes.

8.3 Cell adhesion mediates gut formation

The other major tissue in the sea urchin embryo that undergoes extensive movements and rearrangement is the endoderm. Unlike the PMC and SMC populations, the prospective cells that form the archenteron remain as a cohesive epithelium during gastrulation. This group of cells is initially part of the vegetal plate, one of the primitive territories discussed above, that includes the SMCs, as well as the cells that will form the coelom and other mesodermal derivatives (Cameron *et al.*, 1987). This territory remains as a cohesive group of cells until the SMCs migrate into the blastocoel during the extension phase of gastrulation, and as a consequence the majority of the cells retain their adhesive properties such that they remain associated with their neighbors (Hardin, 1989). The adhesive property of archenteron cells may be an important morphogenetic event as the formation of the gut is independent of influences that stem from the ectoderm or mesoderm. Exogastrula develop morphologically correct tripartate guts outside the embryo (Hardin and Cheng, 1986) and express a gut specific molecular marker (endo16) in a normal spatially regulated manner (Ransick *et al.*, 1993). The SMCs and other mesodermal derivatives that detach from the archenteron lose the ability to express endo16. Therefore adhesion would appear to regulate the expression of this molecule, and endo16 is an ECM molecule that has a RGD binding motif and is intimately associated with gut formation (Soltysikespanola *et al.*, 1994).

The morphogenetic movements that allow for initial archenteron formation are controversial (Ettensohn, 1984; Burke *et al.*, 1991), however this activity is restricted to the vegetal plate and the archenteron, as isolated vegetal halves of embryos allow for the

initial phase of archenteron formation (Ettensohn, 1984). Antibodies directed against components of the apical lamina disrupt the initial phase of gastrulation (Burke *et al.*, 1991), while disruption of collagen crosslinking results in failure of gastrulation (Wessel and McClay, 1987) suggesting that this process requires interactions with the ECM. The extension of the archenteron during the second phase of gastrulation also would appear to be mediated by adhesive events as cell labelling experiments reveal that extension is driven by convergent-extension like cellular rearrangements (Hardin, 1989). This activity is autonomous to the archenteron as isolated vegetal halves allow for archenteron formation and extension (Ettensohn, 1984), and dissociated archenteron cells sort out and form an epithelial tube when combined with ectoderm (Bernacki and McClay, 1989).

Recent evidence suggests that the establishment of fates for cell populations derived from the vegetal plate stem from cell-cell interactions. Deletion of the archenteron results in respecification of tissue from the vegetal plate such that blastomeres located more animal are converted to vegetal plate fates (Logan and McClay, 1994). This respecification occurs with the correct spatial configuration suggesting that cell-cell interactions define cell fates in the normal embryo. Although the respecification of cell fate results in correct gut and skeletal formation other SMC derivatives are often missing, suggesting that the fate of the vegetal plate cells is highly plastic and that a number of cell fates are determined primarily through a series of hierarchical cell surface interactions. The experiments described above indicate that the cell populations derived from a common origin require cell adhesion events for correct spatial patterning and initial determinative events. These cell populations undergo further cell/cell interactions

after initial cell fate decisions have been made and these are responsible for the progressive restriction of cell fate in these populations.

During the final stage of archenteron extension, the SMCs act to both guide and perhaps pull the archenteron tip into juxtaposition with the ectoderm (Hardin, 1988). During the second phase of archenteron elongation SMCs located at the tip of the archenteron send out numerous filopodial extensions that contact the ectodermal basal lamina. The majority of these attachments are transient, however a number of attachments occurring at a site near the apical plate do not retract. Filopodia attached at this site act to either pull or guide the archenteron to this specific target site (Hardin and McClay, 1990). SMC contact with the target site results in a commitment towards SMC fates as these cells are no longer competent to replace depleted PMCs and begin their characteristic migrations after contact with the target site (McClay *et al.*, 1992). This behavior coupled with the loss of endo16 expression at this point is consistent with adhesion mediated regulation of gene expression, although at this time there is no firm evidence to support this.

8.4 Cell adhesion mediates ectodermal fates

The ectodermal epithelium in echinoderms is bounded by both a basal and an apical ECM. The apical ECM is a complex structure consisting of a variety of proteins that are released upon fertilization from granules stored in the egg, many of which are subsequently synthesized constitutively during later stages of development. Many of these proteins are synthesized at high rates during development suggesting a high

turnover rate, although this has not been associated with any specific function. A number of the apical ECM proteins are known to act in adhesion including hyalin (Fink and McClay, 1985, Adelson and Humphries, 1988), and echinonectin (Burdsal, 1991). There is evidence that antibodies directed against a component of the apical lamina, fibropellin, disrupts development (Burke *et al.*, 1991) although the adhesive role that the fibropellins play is unclear (Lail, 1995). The zygotic synthesis of one apical lamina protein, ectoV, shows localized expression in the oral ectoderm (Coffman and McClay, 1990). While it remains unclear what role the apical ECM plays in morphogenesis there is certainly the potential for a role in adhesion mediated events.

There is little other evidence in the sea urchin for regulation of ectoderm morphogenesis by adhesive processes other than those described earlier for the establishment of the five primitive territories. The sole example defined to date is the formation of the ciliated band that defines the boundry between the oral and aboral ectoderm in the late gastrula. Cell lineage analysis reveals that the cell populations that comprise the ciliated band are not clonal in nature suggesting the location of this structure is derived from intercellular signalling events (Cameron *et al.*, 1993). Despite the limited information available on ectodermal morphogenesis it is important to realize that the matrix the PMCs and SMCs migrate upon is most likely deposited by ectodermal cells. PMCs localize to vegetal-lateral regions and are responding to cues from the ECM at this location. The SMCs also contact precise regions in the animal pole suggesting that there is a site that is specifically recognized by cell surface molecules on the SMCs. Therefore,

the ectodermal basal lamina is regionally specialized and that mesenchymal cells are capable of responding to these specializations.

8.5 Differential expression of ECM components

Many of the ECM components that have been described in vertebrates are found in the ectodermal basal lamina, such as fibronectin (Wessel *et al.*, 1984; DeSimone *et al.*, 1985), laminin (McCarthey *et al.*, 1987; Spiegel *et al.*, 1983; Page *et al.*, 1994), and collagen (Ventkatasen *et al.*, 1986). The mutually exclusive expression of a number of genes along oral vs aboral lines (including the ECM component ectoV) suggests that spatial regulation of cellular adhesion could be mediated by such a mechanism. Two ECM components in the gut basal lamina are expressed in a regionally specified manner during development (Soltysikespanola *et al.*, 1994). In the ectoderm basal lamina there is expression of a carbohydrate epitope associated with the ECM that is localized to the vegetal half of the embryo during gastrulation (Ingersoll and Ettensohn, 1994). The apical ECM also has localized expression patterns such as the apical secretion of chondroitin sulphate during the initial phase of gastrulation (Lane *et al.*, 1993). Binding of concanavalin A to the animal half of the ectodermal basal lamina suggests that there is differential expression or glycosylation of ECM components in this area (DeSimone and Spiegel, 1986). Therefore the evidence for the differential expression of ECM in a localized manner correlates well with migratory cell domains.

8.6 ECM receptors in sea urchin embryos

To date there is little documented evidence for the expression or characterization of cell-cell or cell-ECM receptors in the sea urchin embryo. A single cadherin and an associated catenin have been cloned (Miller and McClay, 1994). The cadherin molecule is expressed in all cells of the embryo, perhaps being up regulated in the vegetal plate as PMCs ingress, and is found at the tip of the archenteron during the final phase of gastrulation. It eventually becomes localized to the left coelomic pouch in plutei. Two α integrins have been isolated as PCR fragments from gastrula stage embryos, although nothing is known of the distribution of these molecules (Susan and Lennarz, 1993). Injection of RGD peptides into the blastocoel of sea urchin embryos has been reported to disrupt the migration of mesenchyme cells (Katow *et al.*, 1990). While there is plenty of evidence for cell surface mediated adhesion acting in morphogenesis, pattern formation and determination events, there is little evidence of the specific molecules mediating these events.

9.0 Summary

The integrins are members of a large family of transmembrane receptors that mediate a number of adhesion events during development. Expression is correlated with pattern formation, determination and gross morphological changes. The mechanisms operating in these situations are difficult to assess in vertebrate embryos due to internal development, complex structure, and the inability to visualize internal embryonic structures. The sea urchin embryo provides a model system for the examination of

morphogenetic events. Experimental embryology and biochemical approaches suggest that the molecular mechanisms mediating morphogenesis, pattern formation, and determination events in the sea urchin embryo are similar to those that operate in other organisms. Therefore it is hypothesized that the characterization of cell adhesion molecules in this system will provide a simple model system for the delineation of the events that are mediated through adhesion molecules. To this end I have examined the expression of the β integrin subunits during early sea urchin embryonic development.

MATERIALS AND METHODS

1.0 Embryo culture

Adult *Strongylocentrotus purpuratus* were collected locally and maintained in a circulating sea water system at the University of Victoria. Gametes were collected from adults by intracoelomic injection with 0.55 M KCl. Sperm was collected dry and stored in microfuge tubes at 12° C. Spawned eggs were washed three times in filtered sea water (FSW), strained through 116 µm nitex mesh and left to settle at 12° C. Washed eggs were resuspended, fertilized with diluted sperm and left to settle before being washed four times in FSW. Fertilization success was estimated for each batch of eggs using the elevation of the fertilization envelope as an indicator. If the fertilization rate exceeded 80% the eggs were kept as a monolayer in large embryological bowls in FSW at 12° C. Hatched embryos were decanted, collected by centrifugation and kept in battery jars in FSW. Sea water was changed daily for cultures kept longer than 24 hours.

2.0 Cell Adhesion Assays

Substrates for cell adhesion assays were prepared using either Pronectin-F or Beta-Silk (Protein Polymer Technologies Ltd.,). To create a hydrophobic surface that allows for attachment of the substrate 12 mm round glass coverslips were coated with silicone (Sigmacoat: Sigma) by placing coverslips in vertical racks in a vacuum desiccator along with 1 ml of Sigmacoat in a 5 ml beaker. The desiccator was evacuated and left under vacuum until the Sigmacoat had evaporated. Coverslips were removed from desiccator washed three times in Milli-Q water and air dried. Pronectin-F and Beta silk

were resuspended as stock solutions at 10 mg/ml in sterile phosphate buffered saline (PBS). Sequential ten fold dilutions covering 100 µg/ml to 0.01 µg/ml of Pronectin-F and Beta silk were made up in PBS and placed over coverslips in sterile 24 well tissue culture plates (Nalgene). Substrates were allowed to adhere to the coverslips for 12 hours at 4° C, coverslips were then washed three times in PBS and three times in FSW.

Sibling embryos obtained from fertilizations that used the gametes from a single female and male sea urchin were dissociated using the method of Fink and McClay (1985). After dissociation embryos were washed three times in filtered seawater, filtered through 20 µm nitex mesh, pelleted by centrifugation and resuspended in a large volume of FSW. Dissociated cells were allowed to recover at 14° C for 30 minutes. Cells were again pelleted by centrifugation resuspended at a concentration of 10^6 cells/ml and three ml aliqouts were used for each 12 mm cover slip. Cell adhesion assays were performed in 24 well tissue culture plates (Nalgene). Cells were left to adhere to coverslips undisturbed at 14° C for up to 24 hours. Coverslips were gently rinsed in FSW to remove nonadherent cells and cells remaining on the coverslips were fixed in freshly depolymerized 4% paraformaldehyde in FSW for 30 minutes at 14° C. The coverslips were rinsed three times with FSW and stored at 4° C under FSW. Fixed cells were visualized by incubating the coverslips in Rhodamine Phalloidin (Molecular Probes) 0.01 ug/ml in FSW for 30 minutes at room temperature (RT). Cells were rinsed three times in FSW, three times in PBS, and mounted on Celloseal (Fischer Scientific) rings on a microscope slide using n-Propyl Gallate (6.25 g/litre) in 50/50 PBS/glycerol. All

observations and photographs were made using indirect fluorescence. Cell counts were performed on random fields of view.

To demonstrate the specificity of adhesion to Pronectin F soluble peptides were used to inhibit cell adhesion. For these experiments cells were initially preselected on 100 mm sterile tissue culture dishes coated with Pronectin-F at 10 μg/ml as described above. The cells were allowed to adhere for two hours, the dishes rinsed with FSW to remove nonadherent cells, and adherent cells detached in calcium, magnesium free seawater containing 100 mM EDTA pH 8.0 (CMFSW/EDTA) by repeated washings with a pasteur pipette. Cells were pelleted by centrifugation, rinsed five times in FSW, and plated on substrates at low density (10^2-10^3 cells/ml) for up to 24 hours in the presence of soluble peptides. Soluble peptides, GRGES and GRGDS (Telios Pharmaceuticals) were prepared as stock solutions in FSW at 100 mM, and were diluted to 1 mM in the FSW used for plating cells. Adherent cells were fixed and examined as described above.

3.0 Immunoprecipitations

For immunoprecipitations dissociated cells at 10^6 cells/ml were incubated with gentle shaking at 14° C in the presence of Sulfo-NHS biotin (Pierce Biochemicals) at 5 mg/ml in FSW. To stop the reaction the cells were pelleted by centrifugation and resuspended in FSW containing 10 mM Tris-HCL pH 8.0. Cells were washed three times with FSW/Tris, before being pelleted a final time and frozen at -20° C for 2 hours. Membranes were isolated by repeated washing of cells in Milli-Q water in the presence of the proteinase inhibitor AEBSF (aminoethylbenzene-sulfonylflouride, Calbiochem).

Membranes were isolated by centrifugation and stored at -80° C. Membrane extracts were prepared by homogenizing the lysed membranes in 100 mM β,D-octylglucoside in sterile artificial sea water containing 1 mM AEBSF (SSW). Insoluble material was pelleted as described above and the extracts diluted 1:10 with SSW. Ten µl of each antisera was added and the extracts left to incubate at 4° C ON. Ten µl of a 50% slurry of Protein A agarose (Biorad) in SSW was added to the extracts and they were left on a rotator for two hours. A 1 M sucrose cushion in SSW was made in a 1.5 ml microfuge tube, overlaid with the extract and the agarose pelleted at 1000 X g. The supernatant was aspirated and the pelleted agarose beads washed five times with SSW. After aspiration of the supernatant all traces of SSW was removed from the pellet using a small triangle of filter paper. The pellet was resuspended in non-reducing sample buffer and boiled for 3 minutes before being loaded on a 10% SDS-PAGE gel. For detection of labeled bands the gel was transferred to a nitrocellulose membrane as described above, and the membrane treated as for a western blot. The membrane was incubated with avidin conjugated to alkaline phosphatase for one hour in TBS/milk/Tween 20, washed three times as described previously, and developed as described for western blots.

4.0 RNA isolation

Total RNA was isolated from midgastrula stage sibling embryos using a modified procedure based upon that of Chomczynski and Sacchi (1987). Embryos were pelleted from FSW by centrifugation, resuspended in 10 volumes of ice cold TNM (0.25 M sucrose, 400 mM NH_4Cl, 12 mM $MgCl_2$, 25 mM EGTA, 50 mM Pipes pH 6.5), and

pelleted by centrifugation. Embryos were resuspended in lysis solution (4 M Guanidinium isothiocyanate, 25 mM Sodium Citrate, 0.1 M β-Mercaptoethanol) and immediately homogenized with a hand held polytron (Tissue Tearer Inc.). Sarcosyl was added to a final concentration of 0.5%, the solution well mixed by inversion and centrifuged at 1000 X g to pellet insoluble material. One tenth volume of 2 M Sodium Acetate pH 4.5 was added followed by an equal volume of water saturated phenol. The solution was well mixed and 0.2 volumes of chloroform/isoamyl alcohol (49:1) was added. The solution was mixed well and placed on ice for 15 minutes and subsequently centrifuged at 10,000 X g for 20 minutes at 4° C. The aqueous phase was recovered and mixed with 0.66 volumes of isopropanol and left ON at -20° C. The precipitated RNA was recovered by centrifugation at 10,000 X g for 20 minutes at 4° C, and the pellet redissolved in 1 ml of lysis solution. The RNA was reprecipitated with an equal volume of isopropanol ON at -20° C, collected by centrifugation at 10,000 X g at 4° C, washed in 70% ETOH, pelleted, and dried at room temperature for 10 minutes. The RNA was resuspended in sterile 10 mM Tris-HCl pH 8.0, LiCl was added to a final concentration of 2.5 M from a 10 M stock solution and the RNA precipitated at -20° C ON. The RNA was pelleted by centrifugation at 10,000 X g for 20 minutes at 4° C, washed with 70% ETOH and dried at RT for 10 minutes. The RNA was resuspended in sterile TE (10 mM Tris-HCL pH 8.0, 1 mM EDTA), purity and yield were estimated spectrophometrically using A260 and A280 readings. The RNA was reprecipitated using 0.15 volumes of 2 M Sodium Acetate pH 4.5, and 2 volumes of 100% ETOH at -20° C. All RNA preparations were stored as precipitates at -20° C until used.

Poly A+ fractions were prepared on an oligo-dT cellulose (New England Biolabs) column following the procedure of Kingston (1993). The final pellet was resuspended in sterile water and quantified using A260. The poly A+ fraction was stored at -20° C as a precipitate as described above.

5.0 Polymerase chain reaction (PCR) amplification of β integrin subunits

Degenerate primers for PCR were synthesized at the Regional DNA Synthesis Lab at the University of Calgary. These primers are identical to those described by Erle *et al.*, (1991). For first strand cDNA synthesis, two µg aliqouts of poly A+ RNA from mid-gastrula stage embryos was retrieved from the precipitated stock, collected by centrifugation, washed with 70% ETOH and allowed to dry 10 minutes at RT. The RNA was resuspended in 8.5 µl of 0.22 µm filtered Milli-Q water and 100 ng (in 2µl) of random hexamers (DNA synthesis lab University of Calgary) added. The mixture was heated to 55° C for 10 minutes and cooled to RT slowly, then placed on ice to allow for primer annealing. The first strand cDNA reaction consisted of 4 µl of 5X Superscript II buffer (Gibco/BRL), 2 µl of 0.1 M Dithiothriotol (DTT, Boehringer-Mannheim), 10 units of RNasin (Pharmacia), 2 µl of 10 mM dNTPs in 10 mM Tris-HCl pH 7.4 (Pharmacia), and 200 units of Superscript II MMLV RNase H- reverse transcriptase (Gibco/BRL). The reaction was well mixed, centrifuged briefly and incubated at RT for 15 minutes before being placed at 42° C for one hour. The reaction was heated to 95° C for 15 minutes to inactivate the reverse transcriptase, and 20 units of RNase H (Gibco/BRL) was

added and the reaction returned to 37° C for 15 minutes. The resulting first strand cDNA was used immediately in PCR amplification.

The PCR reaction consisted of 5 µl of the first strand cDNA reaction, 1 µg each of the forward and reverse primer, 1.5 mM $MgCl_2$, 10 µl PCR reaction buffer (Promega), 0.1 mM dNTPs, water up to 100 µl. The reaction mixes were overlaid with 70 µl of Chill Out (MJ Research) and the PCR reactions performed using a Temp-tronic thermocycler (Thermolyne). The first cycle consisted of an initial 5 minute denaturation at 95° C after which 2.5 units of Taq polymerase (Promega) was added, followed by an annealing temperature of 53° C for 2 minutes, and an extension temperature of 72° C for 1 minute. The first cycle was followed by 30 cycles of 94° C, 1 minute; 53° C, 2 minutes; 72° C 1 minute. The final cycle included a 10 minute extension time to polish all product ends. Ten µl of the PCR reaction was separated on a 1.5 % agarose TAE gel to judge product size and yield obtained in the reaction. Reactions producing a single band were glass purified to remove primers and single stranded products using a Sephaglass purification kit (Pharmacia). The resulting product was ligated directly into the pGEM-T vector (Promega), and plated out ON at 37° C on LB agar in the presence of 100 µg/ml ampicillin (Boehringer-Mannheim), and 50 µl of 50 mg/ml X-Gal (Diagnostic Chemicals), and 35 µl of 35 mg/ml IPTG (Biorad). White colonies were selected regrown in LB media ON at 37° C in the presence of 50 µg/ml ampicillin, and plasmids isolated using a Wizard miniprep kit (Promega). Plasmids were sequenced as described below using the SP6 and T7 primers.

6.0 Synthesis of hybridization probes

Probes were synthesized from PCR products using a PCR amplification protocol. For labelling 100 pg of each PCR product was amplified in a 20 µl reaction containing 2 µl of 10X PCR reaction buffer, 1.5 mM $MgCl_2$, 1 µl dNTP mix (60 µM each dGTP, dATP, dTTP), 5 µl of α ^{32}P dCTP (Amersham), 1 µl forward and reverse primer (5 µM each), and 2.5 units Taq polymerase (Promega), for 30 cycles (each cycle consisted of a 1 minute denaturation step at 94° C, 1 minute annealing step at 50° C, and a 30 second extension step at 72° C). After amplification the mix was diluted to 100 µl with TE, and purified through a 1 ml Sephadex G-50 (Pharmacia) spin column constructed in a one ml syringe. Incorporated radioactivity was determined by spotting 1 µl of the diluted reaction on a glass fibre filter before and after purification. Filters were counted in 7 ml. scintillation vials using Ready Safe scintillation cocktail (Beckman) in a Beckman LS6000 scintillation counter. Incorporation was typically 80-90% and produced probes with specific activities of greater then $1X10^{11}$ counts/µg.

7.0 Northern blots

Ten ug of total RNA from unfertilized eggs, 16 cell embryos, blastula, mid-gastrula, and pluteus stage embryos was separated on a 1% formaldehyde-agarose gel following the procedure of Brown (1992). A separate lane was run, and excised from the gel, stained with ethidium bromide to allow for localization of the ribosomal RNA bands. After hybridizations the blot was stained with methylene blue to estimate the the equal loading of lanes (Herrin and Schmidt, 1988). The gel was washed two hours in five

changes of sterile water to remove formaldehyde, and then blotted on to a nylon membrane (Nytran+, Schleicher and Schuell) by capillary transfer ON using 10X SSPE. The membrane was washed three times with 2X SSPE and the RNA was crosslinked to the membrane using 2500 μjoules of UV light in a Stratalinker (Stratagene). Membranes were prehybridized at 55° C in 10 mls/membrane hybridization buffer (equal volumes of 14% SDS, 0.5 M Na_2HPO_4 with 4 mls phosphoric acid/litre) for 30 minutes. For hybridizations a total of 10^6 counts/ml probe (see below) was denatured by boiling and added to 5 mls/membrane of hybridization buffer and incubated ON. Membranes were washed at 55° C in 5X SSPE for 10 minutes, two washes in 2X SSPE 60° C 30 minutes each, followed by a final wash at 65° C in 0.1X SSPE for 20 minutes. Wet filters were placed on a sheet of exposed X-ray film, covered in plastic wrap, and exposed to X-OMAT AR film (Kodak) ON in cassettes containing enhancing screens (DuPont). Blots were stripped in boiling 0.1X SSPE, 0.1 % SDS and reused for all β integrin probes.

8.0 Construction of cDNA library

cDNA was made following the procedures outlined in the cDNA Time Saver kit (Pharmacia). Five μg of poly A+ RNA from midgastrula embryos was used in conjunction with random primers at a dilution (1/100 of stock) that optimized the production of cDNA of about 2000 bp lengths. The cDNA was ligated to dephosphorylated lambda ZAP II arms (Stratagene) and packaged using a Gigapack II kit (Stratagene). The primary library was divided into two aliqouts, one of which was

screened by plaque lifts, the other amplified using the XL-1 MRF' *E. coli* strain (Stratagene). Aliqouts of the amplified library were stored at -80° C in 7% DMSO.

9.0 cDNA library screening

E. coli XL-1 blue MRF' cells were grown ON on LB agar plates in the presence of 12.5 µg/ml Tetracycline. A single colony was used to inoculate a 50 ml culture in NZYM broth with 0.2 % v/v Maltose and grown ON at 30° C. The cells were collected by centrifugation, and resuspended in ice cold 10 mM $MgSO_4$ at an OD_{600} of 0.5. An aliqout of the library representing 10^6 PFU was incubated with 12 mls of XL-1 blue MRF' cells at 37° C for 15 minutes before being plated out a density of 3.0 X 10^5 PFU for each 180 mm NZY agar plate. Plaques were allowed to develop for nine hours and the plates placed at 4° C ON. Plaque lifts were performed using Nytran+ membranes (Schleicher and Schuell). The membranes denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized in 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0, and rinsed in 0.2 M Tris-HCl pH 8.0. DNA was cross linked to the wet membranes using 1200 µjoules/cm^2 UV light (Stratalinker, Stratagene). Hybridizations were performed as described above using 5 mls of hybridization buffer/membrane for prehybridizations, and 2.5 mls of hybridization buffer/membrane for ON hybridizations. Positive plaques were picked and phage eluted into 1 ml of SM buffer (10 mM NaCl, 1 mM $MgSO_4$, 20 mM Tris-HCl pH 7.5, 0.01 % gelatin) ON at 4° C, and replated at a density of 10^3 plaques/180 mm plate. Positive plaques were replated and rescreened until all plaques on a plate were recognized by the probe. The pBluescript

plasmid was retrieved from a single positive plaque using the ExAssist help vector as described in the Stratagene kit.

The same initial plaque lifts were screened using all three PCR products. Between screenings the filters were stripped by immersion in boiling 0.1X SSPE, 0.1 % SDS, three times, and then reprobed using the other β integrin PCR products as probes.

10.0 Sequencing stratagies

Clones representing the sea urchin β integrin subunits were restriction endonuclease mapped using enzymes represented in the multiple cloning site of pBluescript. Double digests were performed using all restriction endonucleases that cut within the cDNA. Fragments were separated on a 0.8% TAE agarose gel in the presence of 1.0 ng/ml EtBr at 30 volts/cm gel. From this data restriction endonuclease maps were constructed for each of the four cDNAs. For sequencing overlapping pieces of the cDNAs were subcloned into pBluescript II KS- (Stratagene). The subclones were sequenced using the dideoxy termination method using a Sequenase II kit (USB/Amersham) and ^{35}S-dATP (Amersham). The primers used for the sequencing reaction were obtained from DNA synthesis lab at the University of Calgary, and were identical to the Stratagene T3 and T7 primers. Sequencing reactions were separated on either 6 or 8 % Urea/TBE polyacrylamide gels using a Biorad Sequegen Cell run at 2000 volts, constant voltage. Gels were dried onto Whatman 3MM paper at 80° C without fixation for two hours under vacuum, and exposed to X-OMAT AR film ON at -80° C. All sequences were read by hand, and entered onto either the PC Gene (Intelligenetics) or

Generunner (Hastings Software) analysis programs. All sequence that represented an open reading frame was obtained either as overlapping fragments isolated from separate lambda ZAP II clones, or as sequence from the coding and noncoding strands of a single clone.

For the cDNAs that had no restriction endonuclease sites allowing for subcloning of overlapping fragments it was necessary to obtain nested deletions. Five µg of each plasmid was digested with restriction enzymes that left adjacent 5' and 3' overhangs, extracted with TE buffered 50/50 phenol/chloroform, once with chloroform, and precipitated at -80° C in the presence of 0.1 volumes 3M Sodium Acetate pH 5.2, and two volumes 100% ETOH for 30 minutes. The precipitated DNA was collected by centrifugation at 13000 X g for 20 minutes in a microfuge. The pellet was washed with 70% ETOH, repelleted, and allowed to dry at RT for 10 minutes. Nested deletion were performed as described in the instructions that come with the Exo III/Mung deletion kit from NEB. All enzymes and buffers were purchased from NEB. The nested deletions were transformed into competent JM109 or XL-1 strain *E. coli* cells and plated on LB agar plates in the presence of 100 µg/ml Ampicillin. A total of 10 colonies from each plate were grown up in five ml aliqouts of LB media with 100 µg/ml Ampicillin, and plasmids isolated using the Wizard miniprep system (Promega). To estimate the size of the deletions all clones were digested with the restriction endonuclease Pvu II (NEB) and the fragments separated on a TAE agarose gel as described above. Subclones representing approximately 200-300 bp deletions were selected for sequencing. Primer

walking was used to fill in holes in all sequences. Primers were purchased from Gibco/BRL. Sequence analysis was performed as described above.

11.0 *In situ* localization of β subunits

In situ localizations were performed essentially as described by Harkey *et al.*, (1992) with the changes noted below. Probes for *in situ* localizations were synthesized using linearized plasmid clones. Linearized plasmids were extracted once with TE saturated phenol, chloroform, isoamyl alcohol (25:24:1), once with chloroform, and precipitated ON at -20° C. The linear plasmids were then used to construct sense and antisense probes using T7 and T3 RNA polymerase (Gibco/BRL) respectively. The reaction consisted of 4 µl of 5 X buffer (Gibco/BRL), 2 µl of 0.1 M DTT, 0.5 µl of RNasin (Pharmacia), 2 µl of ribonucleotide mix containing digoxigenin labelled UTP (Boehringer Mannheim), water up to 20 µl and 50 units of RNA polymerase. The reaction was left at 37° C for 2 hours, then 10 units of RNase free DNAse 1 (Pharmacia) was added and the reaction returned to 37° C for 30 minutes. The RNA was precipitated at -20° C ON by adding 10 M LiCl to a final concentration of 2.5 M. The RNA was collected by centrifugation in a microfuge at 13,000 X g for 10 minutes. The pellet was washed in sterile 70% ETOH, pelleted, the ETOH aspirated and the pellet dried at RT for 20 minutes. The pellet was resuspended in sterile water, the concentration of probe determined spectrophometrically at A260, and the RNA reprecipitated in the presence of 2.5 M LiCl. For use, precipitated probe was resuspended and an aliqout containing 500

ng was removed, collected by centrifugation and resuspended at 50 ng/µl in hybridization buffer (see below).

Staged embryos were fixed (2.5% gluteraldehyde, 0.14 M NaCl, 0.2 M phosphate buffer), on ice for two hours. Fixed embryos were collected by centrifugation and washed twice in 10 volumes of 0.3 M NaCl, 0.2 M Phosphate buffer. Washed embryos were dehydrated through a series of ETOH washes (10%, 30%, 50%, 70%) and stored in 70% ETOH at 4° C.

For *in situ* hybridization embryos were rehydrated in five minute washes of 70%, 50%, 30%, and 10% ETOH, before being washed 3 times 5 minutes in PBS (0.2 M NaH_2PO_4, 0.15 M NaCl). Embryos were then washed 3 times in PBST (PBS with 0.1% Tween 20), digested 10 minutes with 5 µg/ml proteinase K in PBST, washed 30 seconds in stop solution (PBST with 2 mg/ml glycine), washed 3 times with PBST before being postfixed in 4% paraformaldehyde in PBS for 30 minutes and finally washed 5 times in PBST. Embryos were introduced into hybridization buffer (50% formamide, 0.6 M NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.5, 500 µg/ml yeast tRNA, 2X Denhardt's solution and 0.1% Tween 20) slowly using 200 µl volumes of 10%, 30%, 60%, 90%, and 100% hybridization buffer in PBST. Embryos were prehybridized in 200 µl hybridization buffer in a 0.5 ml microfuge tubes for two hours at 45° C. Hybridizations were carried out ON at 45° C in heat sealed glass capillary tubes containing hybridization buffer with 0.01 ng/µl probe. Embryos were washed 10 minutes at RT in 100%, 75%, 50%, 25% hybridization buffer/PBST and finally in PBST. Embryos were then washed twice in PBST at 50° C for 10 minutes, followed by two washes in 1X SSPE at 50° C for 30

minutes. Embryos were then rinsed two times in PBST at RT, blocked 60 minutes in PBST with 5% goat serum and 2 mg/ml BSA (antibody solution), before being incubated in antibody solution containing 0.2 units of anti-digoxigenein Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim) for 60 minutes. The embryos were then washed two times 10 minutes in PBST, and once ON in PBST. Embryos were rinsed with PBST, washed three times with 150 mM NaCl, 25 mM Tris-HCL pH 8.0, then three times with development solution (150 mM NaCl, 25 mM Tris-HCl pH 9.5 containing 0.25 mg/ml levamiosole). Embryos were incubated in development solution containing 2.5 µg/ml NBT/BCIP for up to three hours or until colour was observed. Embryos were washed three times and stored in 10 mM Tris-HCL pH 7.5/1 mM EDTA pH 8.0. For observation and photography embryos were cleared in sequentially in 10%, 30 %, 50%, 60%, 75%, 85%, 95%, 100% terpinol (Sigma) in 100% ETOH. Once in terpinol embryos were stored in 24 well tissue culture plates at 4° C.

12.0 Preparation of antisera

Anti-integrin antisera was prepared in New Zealand white rabbits using bacterially expressed fragments of the βG and βL integrin. Sequence representing the ligand binding domain of these integrins was cloned in the pQE 30 series of vectors (Qiagen) and the recombinant protein products expressed in the M15 *E. coli* strain grown in LB broth in the presence of 10 µg/ml ampicillin and 25 µg/ml kanamycin sulphate at 37° C. Bacterial cultures were collected by centrifugation, frozen at -20°C ON. The histidine tagged proteins were purified under denaturing conditions on a nickle agarose column (Qiagen)

and dialyzed against PBS ON. This resulted in a particulate suspension that was used for immunizations. Three hundred μg of each expressed product was combined 1:1 v/v with Freund's complete adjuvant for primary boosts, or with Freund's incomplete adjuvant for subsequent boosts. After four boosts the rabbits were boosted with expressed protein that had been dialyzed against TBS (dialysis against TBS results in soluble protein). Sera was tested on western blots of 20 ng of expressed protein, and rabbits were bled once the titre was greater than 1:2000. IgG fractions were purified from serum using a Protein A agarose column (Pierce) and concentrated to 1 mg/ml in a stirred cell (Amicon) using a YM 10 membrane (Amicon) and stored at -20° C. Preimmune serum was treated in an identical manner.

13.0 Western Blots

For western blots membrane preparations were made from staged sibling embryos. Embryos were collected by centrifugation, resuspended in FSW containing 1 mM AEBSF (Calbiochem) pelleted and frozen at -80° C. Frozen embryos were thawed on ice, and resuspended in Milli-Q water containing 1mM AEBSF. Membranes were collected at 1500 X g, and repeatedly washed in water/AEBSF until the supernatant was colourless, typically ten washes were required before membranes were collected by centrifugation and the supernatant aspirated. Pelleted membrane preparations were stored at -80°C until used. Membrane extracts were made by adding an equal volume of 100 mM β,D-octylglucoside in Tris buffered saline (TBS) to the thawed pellet and homogenizing the membranes with a small hand held pestle on ice. Insoluble material was collected at

13,000 X g in a microfuge and the soluble fraction examined by SDS-PAGE. SDS-PAGE was performed essentially as described by Laemmli (1970) using a 4.5% stacking gel and a 10% separating gel on a Biorad Mini Protean II apparatus using either reducing sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, and 3.5% β-mercaptoethanol) or non-reducing sample buffer (same as reducing sample buffer without β-mercaptoethanol). Gels were run at 200 volts constant voltage until the dye front had run off the gel. Proteins were electrophoreticaly transfered onto nitrocellulose (Schleicher and Schuell) in a Hoeffer Mini Transfor apparatus at 80 volts constant voltage in blotting buffer (25 mM Tris, 192 mM glycine, 15% methanol). Quality of transfer was estimated by staining the blot with 0.2% Ponceau S in 3% TCA. Blots were blocked ON in 5% skim milk powder, 0.1% Tween 20 in TBS (TBS/milk/Tween 20) at 4° C. Primary antiserum was diluted 1:2000 with TBS/milk/Tween 20 and incubated with the blot for two hours. Blots were washed three times 15 minutes in 100 mls of TBS/milk/Tween 20. Secondary antibody (goat anti-rabbit alkaline phosphatase conjugate: Sigma) was diluted 1:25000 in TBS/milk/Tween 20 and incubated with the blot for one hour. The blot was washed three times 15 minutes in 100 mls TBS/milk/Tween 20, three times in alkaline phosphatase development buffer (150 mM NaCl, 25 mM Tris-HCl pH 9.5), before being incubated in development buffer containing 2.5 ug/ml NBT and BCIP. Colour development was stopped by washing in 10 mM Tris-HCL pH 7.4, 1 mM EDTA pH 8.0. All antibody incubations took place in sealed plastic bags. Relative molecular mass was determined using the MW-SDS-Blue kit from Sigma. Replicates of all experiments were performed with preimmune serum. For immunodepletions serum was incubated with

expressed fragments ON at 4°C in TBS/milk Tween 20, and the depleted serum used in westerns as described above.

RESULTS

1.0 Sea urchin embryonic cells adhere to a conserved integrin binding motif

To gain an estimation of the role that integrin mediated adhesion plays in the early morphogenetic events of the sea urchin embryo, cell adhesion assays were performed using an immobilized substrate containing the conserved integrin binding motif GRGDS. Pronectin-F (Protein Polymers Inc.) is a recombinant molecule consisting of 13 consecutive GRGDS binding motifs cloned into the silk fibroin backbone. The structure of silk fibroin is such that the presentation of the GRGDS site resembles that estimated for fibronectin. As a control, the silk fibroin molecule without the conserved binding motif was used (Beta-Silk, Protein Polymer Technologies).

Initial experiments were performed to gain an estimate of the ability of cells isolated from different stages of dissociated embryos to adhere to Pronectin-F. Cells from all stages appeared to adhere, although those cells isolated from early to late gastrula displayed the most consistency (data not shown), and this stage was used for all subsequent adhesion assays. When dissociated cells are plated on to Pronectin-F, they adhere with a distinct time course. The initial attachment rate depends upon the amount of time the cells are allowed to recover from dissociation and this is reflected in an increase in the numbers of adherent cells up to four hours after plating (Figure 3). Counts of adherent cells in random fields of view reveal that an average of 136± 5.7 on Pronectin-F after one hour. This increases to 187±12 cells after four hours, and decreases after 24 hours to 174±18 cells . In contrast, average counts of adherent cells on β-silk are 10±1.4, 12±1.8, 21±1.7 (Figure 3). These results indicate cells isolated from midgastrula

stage embryos adhere preferentially to the substrate containing the conserved integrin binding motif. The adherence of cells to Pronectin-F allowed the selective purification of cellular populations that bind this substrate. As fewer than 5% of the total number of cells isolated from dissociated embryos bind to Pronectin-F, cells that adhere were preselected before being used in assays. To show that the adhesion was specific to the RGD motif preselected cells were put through adhesion assays in the presence of various peptides that mimic the binding motif. It was evident that the adherent cells consisted of a mixed population (Figure 5) and that the effects of inhibitory peptides was reflected not in adhesive abilities but rather on the ability of these cells to spread and migrate on the substrate. As a result of this the inhibition of spreading was used as a standard to measure the effects of exogenously added peptides. On Pronectin-F 36.5±6.4% of the preselected cells are spread (Figure 4). The addition of RGES peptides that are not inhibitory to the attachment of integrins to the GRGDS site (Hayman *et al.*, 1985) results in 38.3±7.6% of the cells being spread (Figure 4). Addition of a GRGDS peptide results in a decrease in the numbers of cells that spread on Pronectin-F such that only 9.1±2.1% of the cells exhibit a spread morphology (Figure 4). These relative ratios of inhibition are maintained at four hours post plating. It appears that the cells overcome the inhibition of the peptides by 24 hours as 26±8.3% of the cells appear spread at this time (Figure 4).

The cells that attach to Pronectin-F are heterogeneous in appearance and behavior (Figure 5). There are a number of cells that remain rounded although well attatched to the substrate. A large portion of the cells display large lammelipodia and appear to be actively migrating, while other exhibit a characteristic spindel-shaped morphology. Upon

Figure 3: Cells from mid-gastrula stage embryos adhere to Pronectin-F.

Cells from dissociated mid-gastrula stage embryos were plated on Pronectin-F (ProF), or Beta-Silk (Bsilk) substrates for times indicated. Numbers of attatched cells represents the average of counts from 10 random fields of view. Error bars indicate the standard error of the mean.

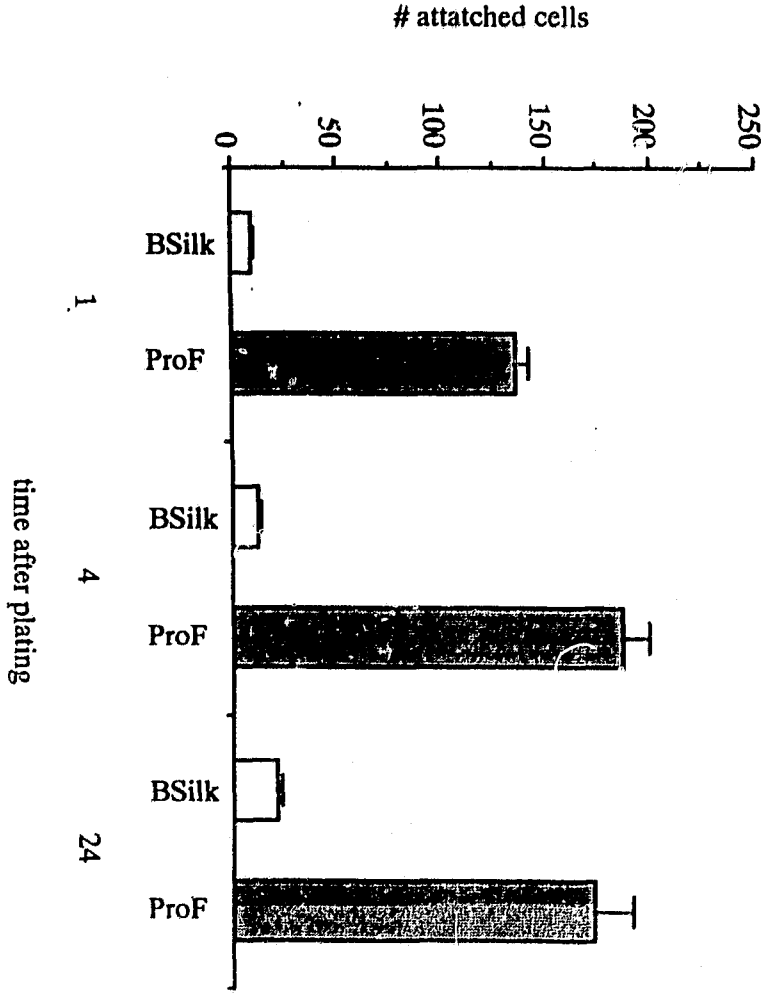
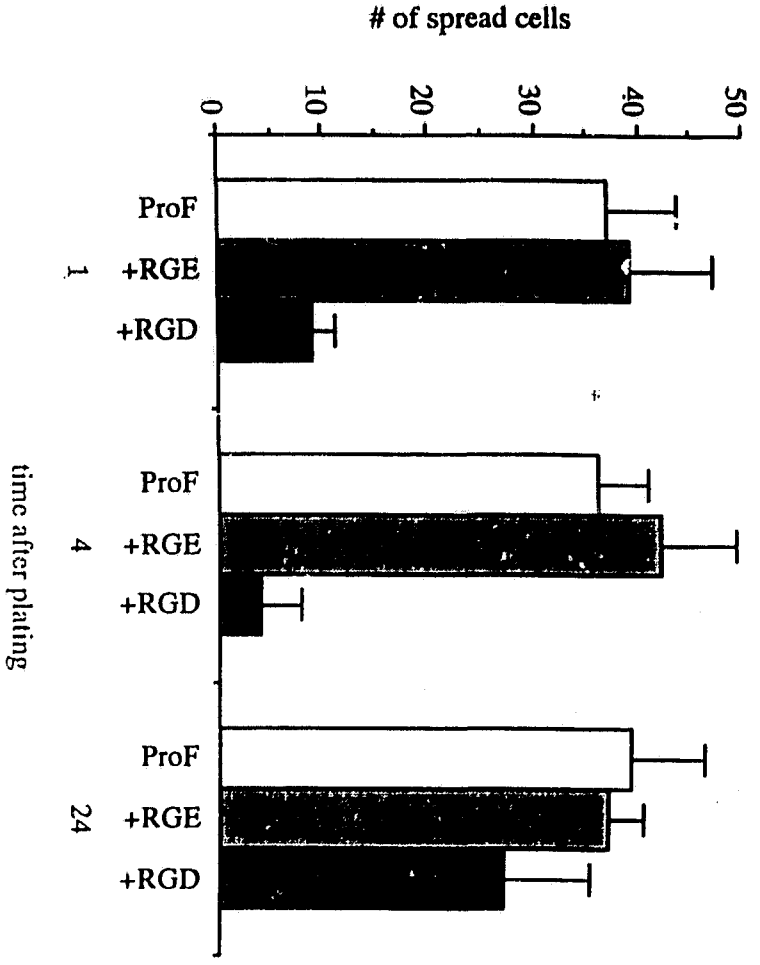

attatched cells
0
50
100
150
250
BSilk
ProF
BSilk
ProF
BSilk
ProF
1
4
24
time after plating

Figure 4: Cells from mid-gastrula stage embryos utilize the RGD sequence to spread on Pronectin-F.

Figure plots percentage of adherent cells spread on Pronectin-F. Cells from dissociated mid-gastrula stage embryos were preselected on Pronectin-F substrates before being plated in the presence of soluble peptides. ProF indicates preselected cells plated in the absence of peptide. +RGE indicates cells plated in the presence of 1 mM GRGES peptide. +RGD indicates cells plated in the presence of 1 mM GRGDS peptide. Percentage spread cells was calculated from 10 random fields of view. Error bars indicate standard error of the mean.


# of spread cells
0
10
20
30
40
50
ProF
+RGE
+RGD
1
ProF
+RGE
+RGD
4
ProF
+RGE
+RGD
24
time after plating

addition of GRGES peptides there is no change in the form of the cells or the relative number of cells exhibiting these forms suggesting that this peptide has no effect on the adhesive abilities of these cells (Figure 5). Addition of the inhibitory peptide GRGDS does not cause a decrease in the relative numbers of adherent cells, however, the cells become rounded lose their spread form and presumably no longer migrate (Figure 5).

The attached cells exhibit a number of features that are revealing to the adhesive interactions taking place. A number of the actively migrating cells are extremely large (Figure 6) and staining of the nuclei of these cells with bisbenzamide (Hoescht) revealed that they are multinucleate (data not shown). Other cells indicated that the syncytial structure arises from the fusion of cells and not mitosis (Figure 6). Both the fusing cells and the large migrating syncytia reveal a number of point adhesions that are a characteristic of integrin mediated adhesion (Figure 6). The rounded cells that result from the treatment with the inhibitory peptide GRGDS also exhibit these point adhesions (Figure 6). All the cells examined in the GRGDS treated samples were mononucleate (data not shown).

1.1 Antibodies to adherent cells recognize ectoderm and the archenteron

To gain a better understanding of the cell populations that were adhering to Pronectin-F, adherent cells were detached with EDTA and used to raise monoclonal antibodies against cell surface molecules. The hybridomas that secreted antibodies were screened against whole fixed midgastrula embryos for cell surface localizations, used in

Figure 5: Sea urchin embryo cells attach to Pronectin-F.

A. Cells from mid-gastrula stage embryos plated on Pronectin-F. Three distinct forms are visible (arrows). Rounded cells that do not spread. Bipolar cells that attach and send out polar extensions. Spread cells that exhibit large lammelipodia.

B. Cells plated on Pronectin-F in the presence of 1 mM GRGES peptide. The cells retain the same forms as described in A.

C. Cells plated in the presence of 1 mM GRGDS peptide. The cells do not spread but remain attached to the substrate.

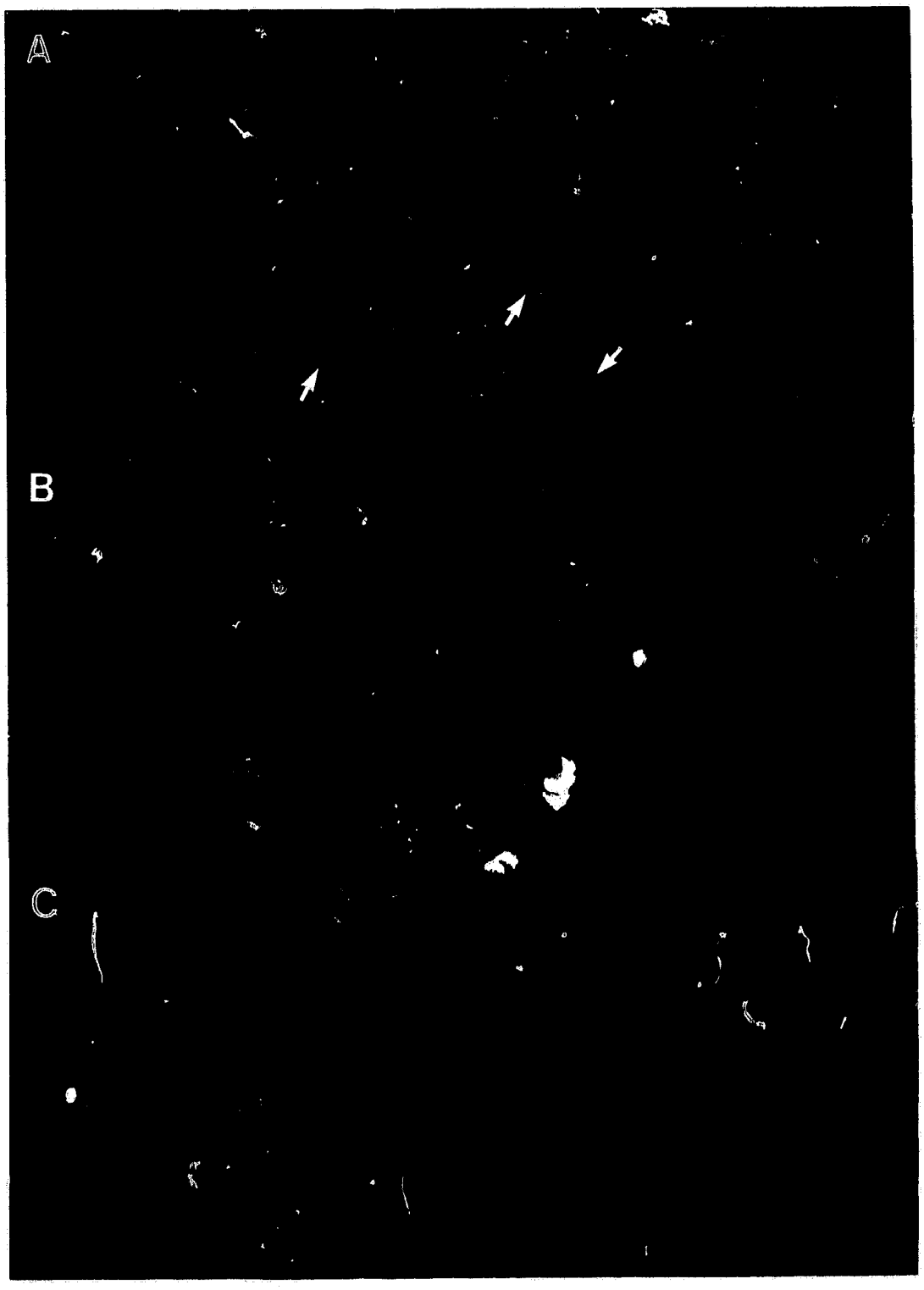

A
B
C

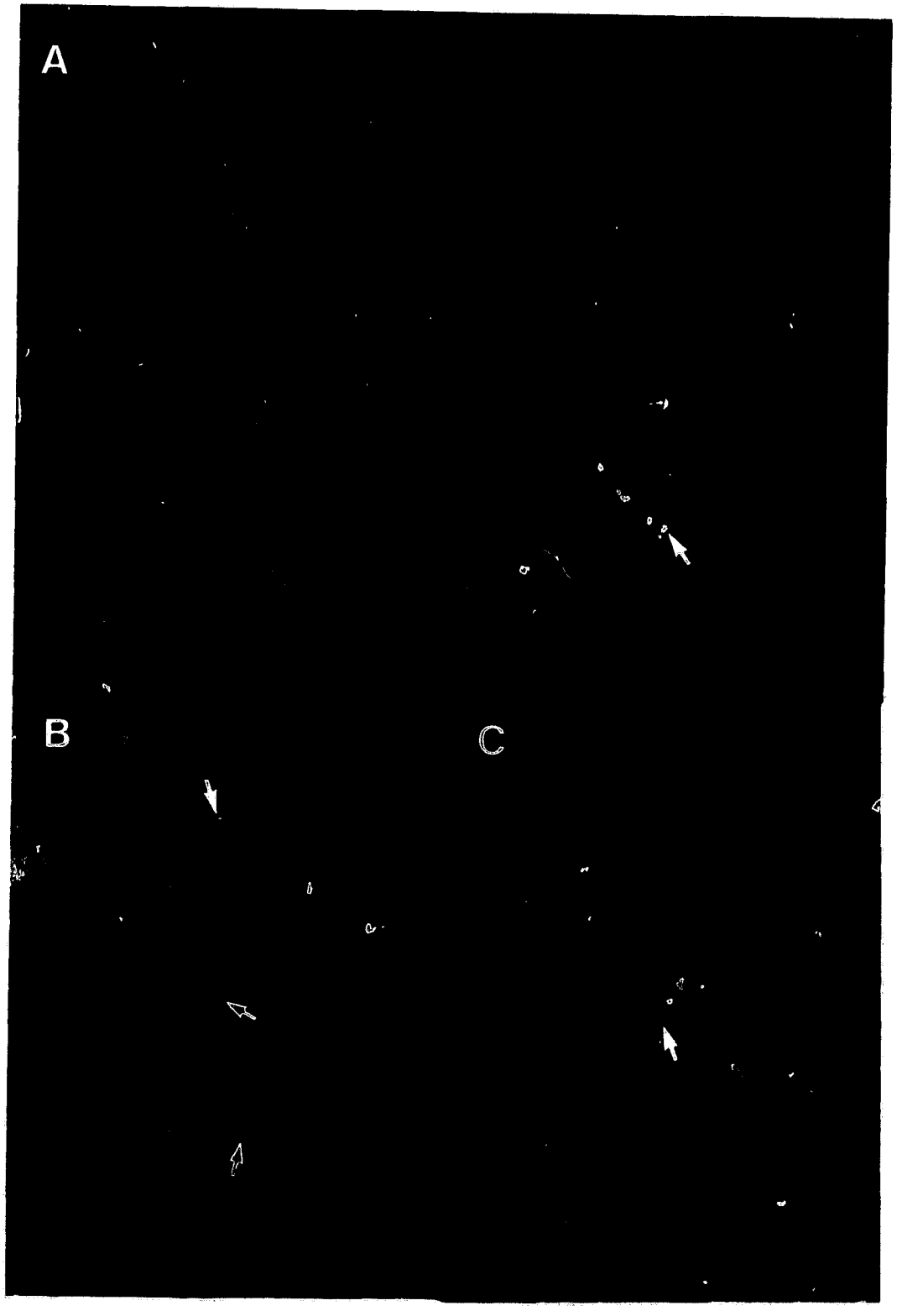
Figure 6: Cells plated on Pronectin-F exhibit unique behaviors.

All cells in the figure have been stained with Rhodamine Phalloidin to visualize the actin cytoskeleton

A. Large multinucleate cell on Pronectin-F substrate. A number of point adhesions are visible (arrows)

B. Two multinucleate cells undergoing fusion. The presence of cells such as these suggests that the syncytial nature of the multinucleate cells is due to fusion rather than karyokinesis. Actin fibrils are visible, as well as a number of point adhesions (arrows).

C. Mononucleate cell on Pronectin-F substrate in the presence of GRGDS peptide. The cell exhibits a number of point adhesions.


A
B
C

immunoprecipitations of cell surface labelled material, and used in western blots. Eleven hybridomas of the 3500 screened met this criteria and were kept for further analysis. Of these, four antibodies which have interesting characteristics are presented here.

When used in immunoprecipitations from cell surface labelled material these antibodies all precipitate a doublet running at approximately at an apparent molecular weight of 73 kDa and 54 kDa (Figure 7). While all the immunoprecipitates appear similar they are not identical. The similarity between samples may actually be a consequence of the screening procedure.

Antibody 2A10 is found primarily in intracellular compartments of epithelial cells (Figure 7). Antibody 8B1 is found on the surface of a large number of ectodermal cells and is distributed in an unpredictable manner (Figure 7). Antibody 4E1 also exhibits an epithelial distribution and appears as patches (Figure 7).

Antibody 8F2 recognizes an epitope that localizes to the apical periphery of all cells in the blastula (Figure 8). As development proceeds the domain of this epitope appears to condense and the signal around the cell periphery sharpens (Figure 8). As gastrulation occurs the epitope is found in the ectoderm and in the archenteron, no staining is observed in mesenchyme derivatives. In postgastrula stages the epitope gradually decreases in abundance on aboral surfaces, while being maintained in the gut and oral surface. In prism stages the staining in the gut is lost while that of the oral ectoderm is retained (Figure 8).

2.0 Cloning of β integrin subunits from *Strongylocentrotus purpuratus*

The known β integrins all contained a highly conserved region that is centered around the binding site. Degenerate primers that were designed against this site (Erle *et al.*, 1991) were used in polymerase chain reaction (PCR) to identify amplification products that were specific to sea urchins. On 2% agarose gels the product appears as a single band (Figure 9). The products were isolated using T-Vector cloning (Promega) and identified by sequencing both strands. Three β integrin products were isolated that bear approximately a 50% similarity to vertebrate β integrin subunits at the amino acid level (Figure 9). Amino acid residues that are conserved in all known integrins are found in the open reading translations of the isolated sea urchin cDNA fragments (Figure 9). Two hundred colonies resulting from a single PCR amplification were analyzed by sequencing and only the three products described were identified. Over 12% of the products identified represented histone 2A gene products and it appears the extensive degeneracy of the primers results in priming of this product. In a single instance a product resembling the βL integrin was identified. This PCR product was 103 bp longer than those shown and did not include an open reading frame that encompassed the whole clone. There was an open reading frame identified that extended from either primer, however this breaks down in the middle of the clone. It is unknown if this represents a legitimate product that is the result of alternative splicing events and is incomplete due to the errors that can be introduced by PCR, or if this is the result of a recombination event

Figure 7: Monoclonal antibodies raised against adherent cells recognize epithelial epitopes.

A. Immunoprecipitations of biotin labelled cell surface molecules. Lane 1 antibody 2A10. Lane 2 antibody 8B1. Lane 3 antibody 4E1. Lane 4 antibody 8F2. All antibody immunoprecipitates consist of two bands that migrate at an apparent molecular weight of 73 kDa and 54 kDa.

B. Immunolocalization with antibody 2A10. 2A10 recognizes scattered epithelial cells (arrow) located in the ectoderm and archenteron. The epitope is primarily cytoplasmic.

C. Immunolocalization with antibody 8B1. 8B1 recognizes scatterred groups of epithelial cells (arrow) in the ectoderm. The epitope is localized to the cell surface.

D. Immunolocalization with antibody 4E1. 4E1 recognizes epithelial cells in the ectoderm (arrow) and at the tip of the archeteron. The ectodermal cells occur mostly as patches, although solitary cells are visible.

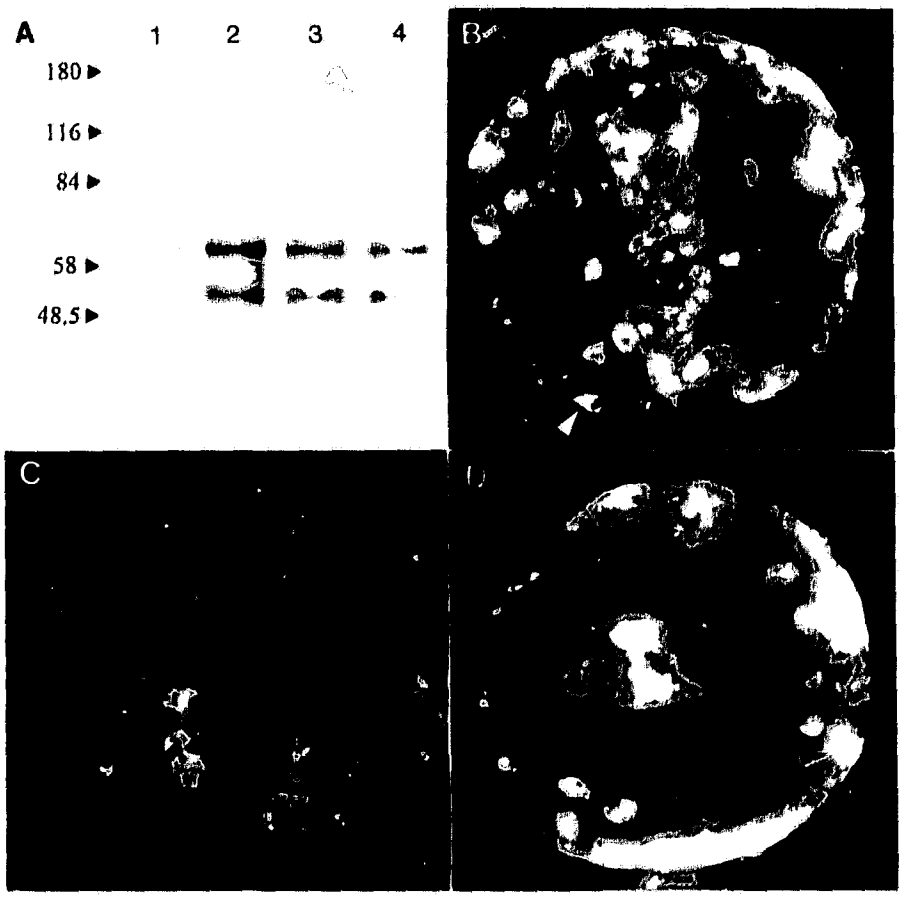

A
1
2
3
4
180
116
84
58
48,5
B
C
D

Figure 8: Immunolocalization of antibody 8F2.

A. 8F2 localizes to the apical periphary of all epithelial cells in the blastula. Staining is more intense at the animal pole.

B. At mid-gastrula 8F2 is localized to the periphary of all epithelial cells, including those of the archenteron. Staining is strongest the animal pole and at the lateral margins of the archenteron.

C. Surface view of the same embryo as in B.

D. Prism stage embryo (lateral view). 8F2 expression is decreasing in the aboral tissues (arrow), but continues to be expressed at high levels in the oral face and archenteron.

E. Pluteus (ventral view). 8F2 expression is high in the oral face and digestive tract.

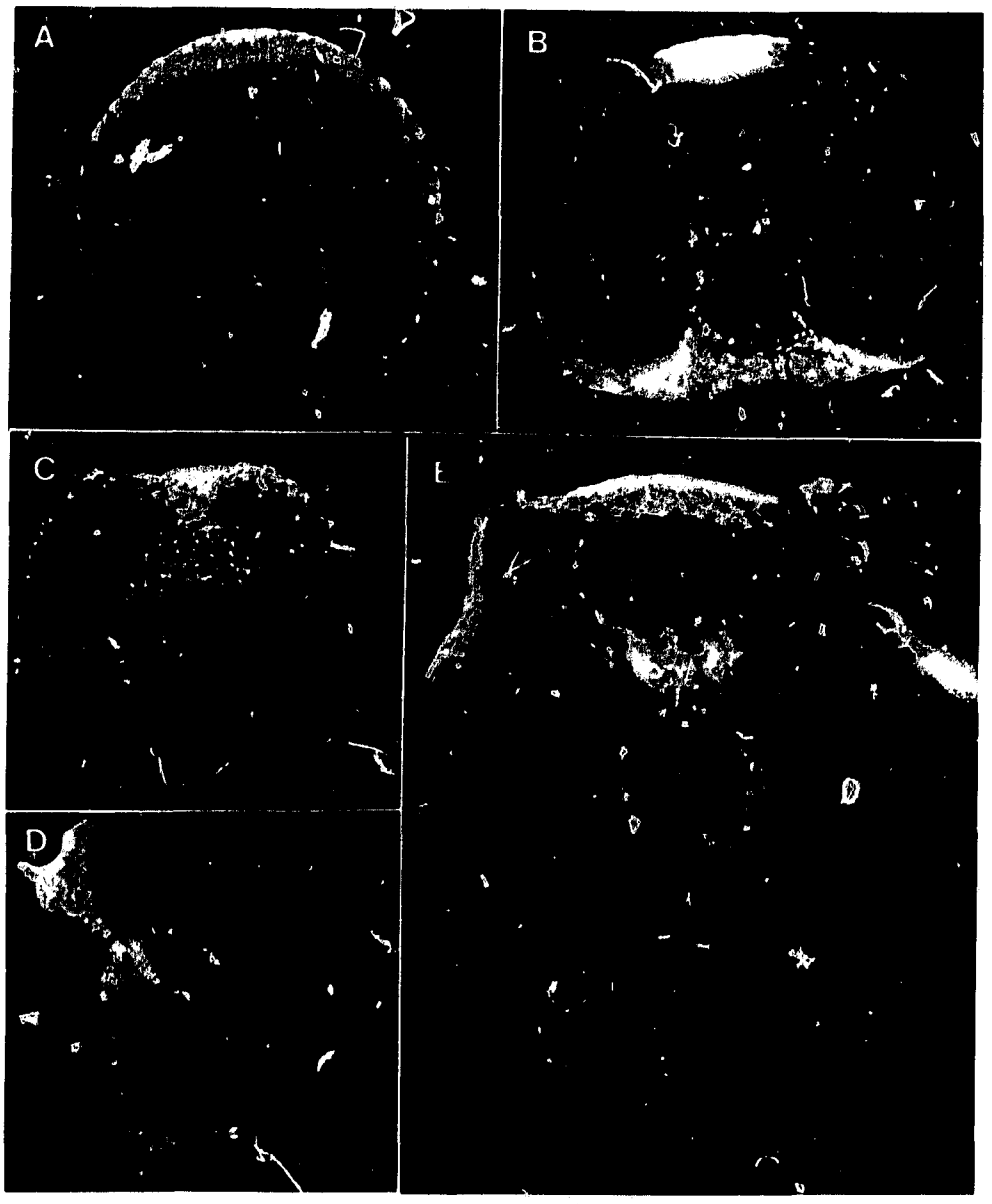
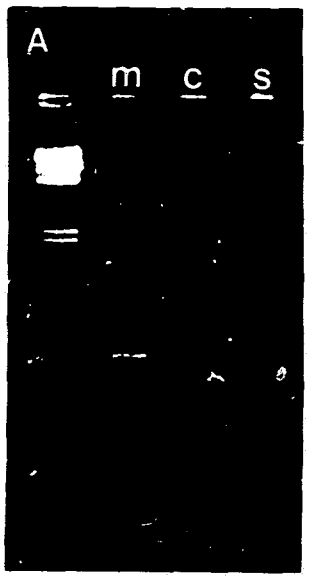

A
B
C
D

Figure 9: PCR amplification of sea urchin β subunits

A. Ethidium bromide stained agarose gel. Lane 1 Lambda phage Genomic DNA digested with Hind III. Sizes of fragments from top of gel in bp are 231330, 9416, 6557, 4361, 2322, 2027, 564. Lane m PCR product from mouse cDNA β1 clone (350 bp). Lane c PCR product from cDNA made from chick embryo poly A+ RNA (350 bp). Lane s PCR product from sea urchin embryo poly A+ RNA (350 bp).

B. Alignment of deduced amino acid sequence of the three sea urchin β1 cDNAs isolated by PCR. The human β1 integrin are used to represent the eight known families of β integrin subunits are represented at the top of the figure (HUMβ1-HUMβ8). The three sea urchin cDNA clones are represented at the bottom of the figure (SpβC, SpβG, SpβL). The verticle lines represent residues that have conservative substitutions in the majority of subunits and the asterisks represent sites that are completely conserved.



B

```
HUMβ1       MKDDLENVKSLGTDLMNEMRRITSDFRIGFGSFVEKTVMPYISTTP-AKLRNPC-TSE--QNCTTPSGYKNVLSLTNKGEVFNELVGKQRISGNLDS
HUMβ2       MLDDLRNVKKLGGDLLRALNEITESGRIGFGSFVDKTVLPFVNTHP-DKLRNPCPNKE--KECQPPFAFRHVLKLTNNSNQFQTEVGKQLISGNLDA
HUMβ3       MKDDLWSIQNLGTKLATQMRKLTSNLRIGFGAFVDKPVSPYMYISPPEALENPC--YDMKTTCLPMFGYKHVLTLTDQVTRFNEEVKKQSVSRNRDA
HUMβ4       MSDDLDNLKKMGQNLARVLSQLTSDYTIGFGKF-DKVSVP-QTDMRPEKLKEP------WPNSDPPFSFKNVISLTEDVDEFRNKLQGERISGNLDA
HUMβ5       MKDDLDNIRSLGTKLAEEMRKLTSNFRLGFGSFVDKDISPFSYTAPRYQ-TNPCIGYKLFPNCVPSFGFRHLLPLTDKVDSFNEEVRKQRVSRNRDA
HUMβ6       MDDDLNTIKELGSGLSKEMSKLTSNFRLGFGSFVDKPVSPFVKTTP-EEIANPCSSIPYF--CLPTFGFKHILPLTNDAERFNEIVKNQKISANIDT
HUMβ7       MKDDLERVRQLGHALLVPLQEVTHSVRIGFGSFVDKTVLPFVSTVP-SKLRHPCPTRL--ERCQSPFSFHHVLSLTGDAQAFEREVGRQSVSGNLDS
HUMβ8       MHNNIEKLNSVGNDLSRKMAFFSRDFRLGFGSYVDKTVSPYISIHP-ERIHNQCSDYNL--DCMPPHGYIHVLSLTENITEFEKAVHRQKISGNIDT

consensus   MKDDLDNVKKLGTDLARELRELTSNFRIGFGSFVDKTVLPFVSTTP-EKLRNPCPNRENFPNCQPPFSFKHVLSLTNDADAFNEEVGKQRISGNLDA
            * |.|  |  |*  *   |   | |  |*|* | |*   *     |      |         | |  || | | *|     *   |     * * *

SpβC        MSDDLVQLRSLGGILAGEVKNITNNFRLGYGAFIDKTVMPSVDIYP-AKLENPCLNK----RCGPAFSFHNILPLTLETDRITEEISKVNSSGNLDI
SpβG        MKDDLENLKGLGTTLSEELNSITRDFRLGFGSFVDKTVLPYVSTVP-AKLISPCTG------CASPHGFHNALPLNQDPSLFANRITNTTVSGNLDT
SpβL        MEDDLSKLMDLGDILASEMKNITSNFRLGFGSFVDKTVMPYVSTVP-EKLIAPCTG------CEAPYGFKNVLPLNENTNLFSETVMNQRASGNLDA
```

or perhaps errors occurring in the cDNA synthesis or during amplification. This clone was not characterized further.

In an attempt to identify other β integrins a sample of the band isolated in the initial PCR reaction was cut with restriction enzymes specific for each product identified. A sample of the digested DNA was used in a subsequent PCR reaction and the products cloned and analyzed by sequencing. Of 60 clones isolated and sequenced all represented histone gene products and the method was abandon.

The PCR clones were used to probe Northern blots to gain an estimate of the time of peak expression for each clone. The PCR products all displayed distinct temporal peaks in expression (Figures 12, 15, 21). These clones were named after the times in development at which expression is highest. As the βG (beta gastrula) and βL (beta larval) integrins were expressed highly at the gastrula stage of development, and the βC (beta cleavage) subunit was expressed at low levels, a random primed cDNA library was constructed from poly A+ RNA isolated from midgastrula stage embryos and screened before amplification with each of the PCR products.

2.1 The βC integrin subunit

2.11 Primary sequence and deduced amino acid sequence of the βC subunit

The βC probe identified only a single plaque of the 10^6 plaques screened and this clone was isolated and sequenced (Figure 10). The clone is incomplete and represents an area that encompasses the 5' end of the ligand binding domain up to a region that ends just proximal to the transmembrane portion of the integrin subunit. The fragment of βC

Figure 10: Map of sea urchin βC subunit cDNA

A schematic of the predicted protein is aligned with the cDNA obtained from a midgastrula stage cDNA library (βC 1). For the protein sequence the PCR fragment is indicated by a speckled region. Restriction enzyme sites used to generate subclones are indicated. Subclones used for sequencing are named after the original clone and the restriction enzyme(s) used to isolate specific fragment. All subclones were sequenced from either end using T7 and T3 primers.

Beta C seq 1595 base pairs

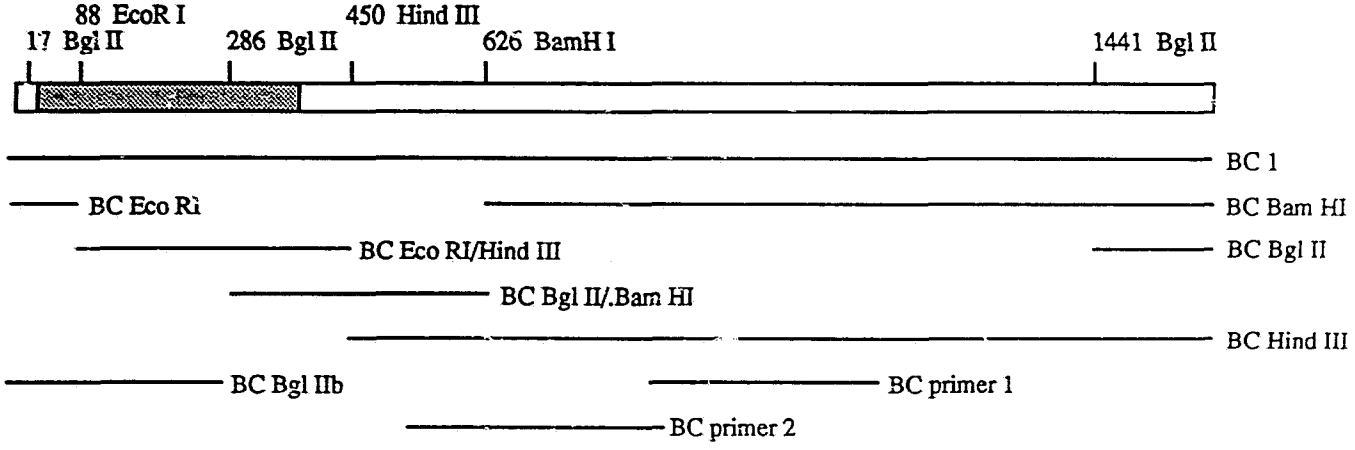


Figure 11: Nucleic acid sequence and predicted amino acid sequence of the sea urchin βC subunit

Cysteines are in bold and numbered as described in the text. The cysteine residues are numbered starting with cysteine 8 as the first 8 cysteine residues are conserved in all β integrin subunits. Potential N-glycosylation sites are indicated with an asterisk.

```
  1 ATG GCC GAG GAC TTC CCT GTA GAT CTT TAC TAC CTT ATG GAC TTG   45
  1  M   A   E   D   F   P   V   D   L   Y   Y   L   M   D   L   15

 46 TCA GAC TCC ATG TCT GAC GAC TTG GTC CAA CTT AGA TCA TTG GGT   90
 16  S   D   S   M   S   D   D   L   V   Q   L   R   S   L   G   30

 91 GGA ATT CTA GCT GGT GAA ATG AAG AAC ATC ACC AAT AAT TTC AGG  135
 31  G   I   L   A   G   E   M   K   N   I   T   N   N   F   R   45
                                   *
136 CTG GGC TAC GGT GCC TTT ATT GAT AAA ACC GTT ATG CCT TAC GTT  180
 46  L   G   Y   G   A   F   I   D   K   T   V   M   P   Y   V   60

181 GAT ATC TAC CCA GCA AAG TTA GAG AAT CCA TGT TTG AAT AAG AGG  225
 61  D   I   Y   P   A   K   L   E   N   P   C   L   N   K   R   75
                                           8
226 TGT GGT CCA GCA TTC TCC TTC CAT ATT CTT CCA CTC ACG CTT GAG  270
 76  C   G   P   A   F   S   F   H   I   L   P   L   T   L   E   90
     9
271 ACA GAT AGA TTT ACT GAG GAG ATC TCT AAG GTT AAT AGT TCT GGT  315
 91  T   D   R   F   T   E   E   I   S   K   V   N   S   S   G  105

316 AAC CTT GAC AGC CCT GAA GGT GGT ATG GAC GCT CTA ATG CAA GCC  360
106  N   L   D   S   P   E   G   G   M   D   A   L   M   Q   A  120
     *
361 ACA GTC TGC ACA GAT GAG ATT GGC TGG AGG GTG TGT GCC AGG CAT  405
121  T   V   C   T   D   E   I   G   W   R   V   C   A   R   H  135
            10                                   11
406 CTG CTT GTG TAC ACC ACT GAT GCT AGC TTC CAC ATC GCT GGA GAT  450
136  L   L   V   Y   T   T   D   A   S   F   H   I   A   G   D  150

451 GGA AAG CTT GGT GGT ATC GTA AAG CCC AAT GAT GGC AAG TGT CAT  495
151  G   K   L   G   G   I   V   K   P   N   D   G   K   C   H  165
                                                       12
496 ATG GAT AGT ACT GGC TTT GAA TAC ACC ATG GCA AAT GAA ATG GAC  540
166  M   D   S   T   G   F   E   Y   T   M   A   N   E   M   D  180

541 TAT CCT TCC ATC AGT AAG CTG AGT CAG AAG ATG GAG ACT CTC ACC  585
181  Y   P   S   I   S   K   L   S   Q   K   M   E   T   L   S  195

586 ATT CTA CCA ATC TTC GCC ATC GGG AAA GCT GAA GTA GAT AAA CAG  630
196  I   L   P   I   F   A   I   G   K   A   E   V   D   K   Q  210

631 GAT CCA TTC GTC TTT TAT GAG GAT CTT CCA CAA TAC TTT CAT GAG  675
211  D   P   F   V   F   Y   E   D   L   P   Q   Y   F   H   E  225

676 TCG AAG GCA GCC AGA CTT TCT GCA GAT TCA TCC AAC ATT GTA GAT  720
226  S   K   A   A   R   L   S   A   D   S   S   N   I   V   D  240

721 TTG ATC AAG AAC ATA TAT TTA AAT ATC ACG TCT GAA GTG ACG GTA  765
241  L   I   K   N   I   Y   L   N   I   T   S   E   V   T   V  255
                                   *
766 GAG ACA AGG CTC GGG GCA GAC CTG TTT GAG GTA GAT TAC GTG GCC  810
256  E   T   R   L   G   A   D   L   F   E   V   D   Y   V   A  270

811 CAC TGT CTA GAC GGC TCC ATT ACC AAG GAT AAA CAG ACC TGT ATG  855
271  H   C   L   D   G   S   I   T   K   D   K   Q   T   C   M  285
                                                       13
856 GGA CTC AAG CTA GGG GAT CAA ATC AGC TTT GAT GTC GGT ATT ACC  900
286  G   L   K   L   G   D   Q   I   S   F   D   V   G   I   T  300
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901 ATG AAG AAT TTG AGC TGC ACA GTT CAA CAA TGG AAT GCA CAC GAT  945
301  M   K   N   L   S   C   T   V   Q   Q   W   N   A   H   D  315
                 *           14
946 TCA GTA GGC CGA CCA GTT TTC ACA GAA AAT CTT GTC CTC AAC GTG  990
316  S   V   G   R   P   V   F   T   E   N   L   V   L   N   V  330

991 AAA GCA CTT TGC GAA TGC GAT TGC TCC AGC AGT GAG CAT GAG CCC 1035
331  K   A   L   C   E   C   D   C   S   S   S   E   H   E   P  345
                15      16      17
1036 AAC TCC ACT AAA TGT AAC TTC CAT GGT ACG TTC ACA TGT GGT GCC 1080
 346  N   S   T   K   C   N   F   H   G   T   F   T   C   G   A  360
      *                18                          19
1081 TGT ATG TGT AAC GAA GGA CGA TCA GGG AGG ACC TGT GAA TGT GAC 1125
 361  C   M   C   N   E   G   R   S   G   R   T   C   E   C   D  375
     20      21                              22      23
1126 AGA GTA GAG GCT GAA GGG CTT GAC CCA AGC TGC GTA CAA CCC AAT 1170
 376  R   V   E   A   E   G   L   D   P   S   C   V   Q   P   N  390
                                          24                  *
1171 TCT ACT GTA GAG TGC TCC AGT AGA GGC ACC TGC GTA TGC GGT GAA 1215
 391  S   T   V   E   C   S   S   R   G   T   C   V   C   G   E  405
                    25                      26      27
1216 TGT GAA TGT GAC ACG CGT GGT GAC CCT AAC CGT ATC ATC ACA GGA 1260
 406  C   E   C   D   T   R   G   D   P   N   R   I   I   T   G  420
     28      29
1261 GAG TAC TGT CAG TGC GAC AAC TAC CTG TGC CCA AGA TCA GGA GGA 1305
 421  E   Y   C   Q   C   D   N   Y   L   C   P   R   S   G   G  435
             30      31                  32
1306 GAG GTG TGC GGA GGT AGT GAT AAA GGA ACA TGT CTT TGC GAT GTT 1350
 436  E   V   C   G   G   S   D   K   G   T   C   L   C   D   V  450
             33                          34      35
1351 GAT GTT GGT AAC TTC TGT GGC TGT TTA GAA GGT TAT GAA GGC AGT 1395
 451  D   V   G   N   F   C   G   C   L   E   G   Y   E   G   S  465
                        36      37
1396 GCT TGT GAG TGC CCT ACA TCT AAT GAT ACC TGT AGA GCT CCA AAC 1440
 466  A   C   E   C   P   T   S   N   D   T   C   R   A   P   N  480
         39      40                          4
1441 GGG GAG ATC TGT AAT GGA GTA CTG TCC TGT GAT TGT GGA AAG TGT 1485
 481  G   E   I   C   N   G   V   L   S   C   D   C   G   K   C  495
                 42                  43      44          45
1486 CAA TGT AAT GAT CCT AAG TAC TCC GGT GCT ACC TGT CAA ATA TGT 1530
 496  Q   C   N   D   P   K   Y   S   G   A   T   C   Q   I   C  510
         46                                      47          48
1531 CCA GAC TGT GCT GGT GAG TGT GAC ATC TTC CAG CCT TGT GTA CAG 1575
 511  P   D   C   A   G   E   C   D   I   F   Q   P   C   V   Q  525
             49                  50                  51
1576 TGT AGA GCC TTC CAC ACC GGA GCT                         1599
 526  C   R   A   F   H   T   G   A                          533
     52
```

isolated encodes a 1595 bp open reading frame that encodes a 531 amino acid protein (Figure 11). There are seven potential N-glycosylation sites within this fragment (Figure 11). No attempts were made to isolate other fragments as the likelihood of obtaining another fragment representing this molecule from the library are small. The peak expression of this molecule occurs during the cleavage stage of development and the prevalence of this molecule has decreased by the stage from which the library was made (Figure 12).

2.12 Comparison of the βC subunit to other β integrin subunits

When compared against human integrin subunit fragments that represent similar fragments of the β subunit, the βC subunit has approximately 26% identity (Table 2). This is similar to that found when this fragment is compared to the other sea urchin β subunits (30% for βG and 26% for βL).

The cloned fragment of βC contains 44 of the 56 cysteine residues that are found in most β integrin subunits. Those cysteine residues that are present are in conserved positions with the exception of the cysteine at position 11 (Figure 26). A cysteine in this position is not found in any vertebrate β integrin subunit. The other sea urchin β integrin subunits also do not have cysteine residues in this location (Figure 26).

2.13 Temporal patterns of βC expression

The βC subunit is expressed in the unfertilized egg as a 7.5 kb transcript and increases in prevalence from fertilization up until the cleavage stages when it becomes less

Table 2: Identities among integrin β subunits.

The percentage identity between the extracellular domains of the sea urchin β subunits and the human β1-β8 subunits were computed using the Gene Works program (Intelligenetics). For comparisons to the SpβC subunit only sequences that aligned with the cloned fragment were used to calculate % identity.

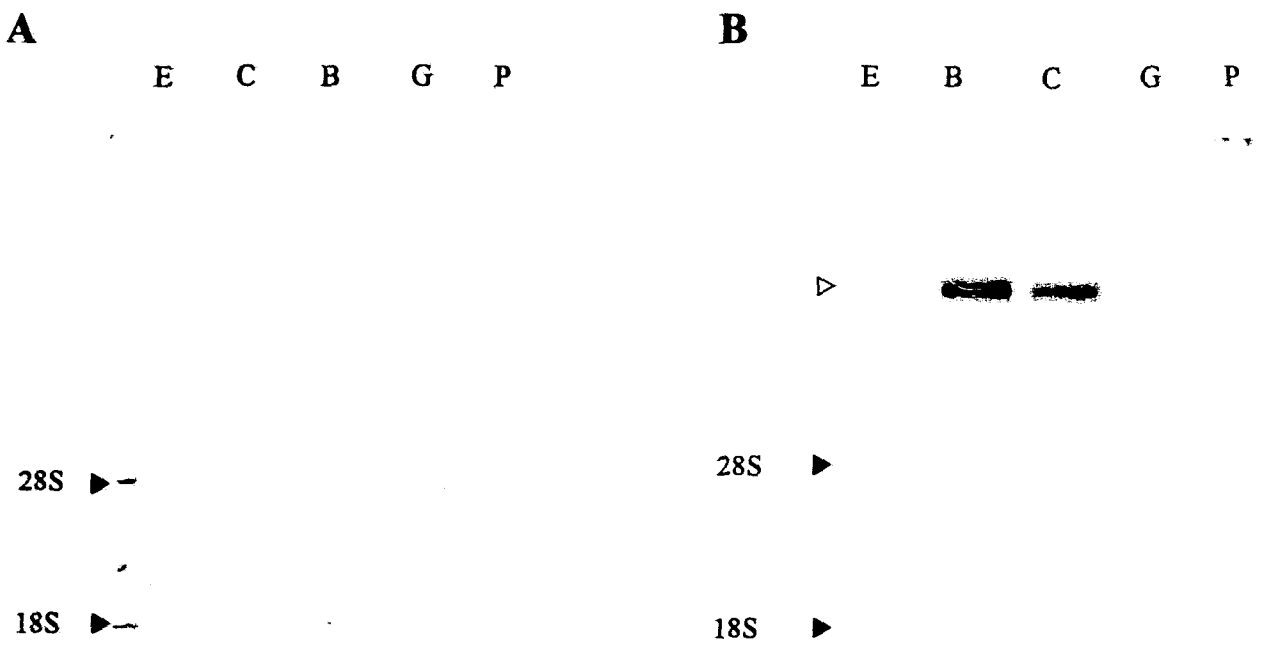
	β1	β2	β3	β4	β5	β6	β7	β8	βC	βG	βL
βC	29	26	27	11	27	26	24	21	-	30	26
βG	40	33	38	29	36	36	34	27	30	-	43
βL	40	33	38	16	35	35	35	28	26	43	-

Figure 12: Northern blot analysis of βC

Northern blots were performed on 10μg of total RNA isolated from egg (E), cleavage (C), blastula (B), gastrula (G), and pluteus (P) stage embryos.

A. Methylene blue stained membrane showing localization of 28S and 18S ribosomal RNAs.

B. The same blot as in A hybridized with a probe made from the βC PCR product. A single band of 7.5 Kb is visible at all stages βC expression peaks in the cleavage and blastula stages. The size of the βC transcript was estimated from the rRNAs.


A
E C B G P
28S
18S
B
E B C G P
28S
18S

abundant in the blastula stage (Figure 12). There is evidence of the transcript at all stages of development but the level of expression is assumed to be low even at the cleavage stage due to the weak signals obtained on the northern blot.

2.2 The βG integrin subunit

2.21 Primary sequence of the βG subunit and deduced amino acid sequence

The βG subunit was isolated as a total of four overlapping cDNA fragments (Figure 13) that encompass the coding sequence and approximately 2.5 Kb of 5' and 3' untranslated sequence (Figure 14). The 2577 bp coding sequence encodes a 799 AA open reading frame with a predicted molecular weight of 90 kDa (Figure 14). A potential signal peptide consisting largely of hydrophobic residues follows the first usable ATG codon and ends with a predicted cleavage site at the serine residue at position 29 (von Heijne, 1986). As depicted in Figure 14 there are eight predicted N-glycosylation sites in the deduced extracellular domain. A predicted 28 amino acid transmembrane domain extends from residue 715 to residue 743 (Figure 14). The presumptive cytoplasmic domain of the βG subunit is 42 amino acids long (Figure 14). The 3' untranslated sequence does not contain a polyadenylation signal within the region sequenced.

2.22 Comparison of the βG subunit to other β integrins

The βG subunit has approximately a 35% identity with the human β integrin subclasses (Table 2). The highest identity is with the human β1 subunit (40%) although this is not much less than the identity with the other sea urchin integrin subunits (Table 2,

Figure 13: Map of the sea urchin βG subunit cDNAs and sequencing strategy

A schematic of the predicted protein is aligned with cDNAs obtained from a midgastrula stage cDNA library (BG 11, BG 13, Bg 14, Bg 15). For the protein sequence the PCR fragment is indicated by a speckled region and the transmembrane domain by a hatched region. Restriction enzyme sites used to generate subclones are indicated. Subclones used for sequencing are named after the original clone and the restriction enzyme(s) used to isolate that fragment. All subclones were sequenced from either end using T7 and T3 primers.

Beta G seq 2578 base pairs

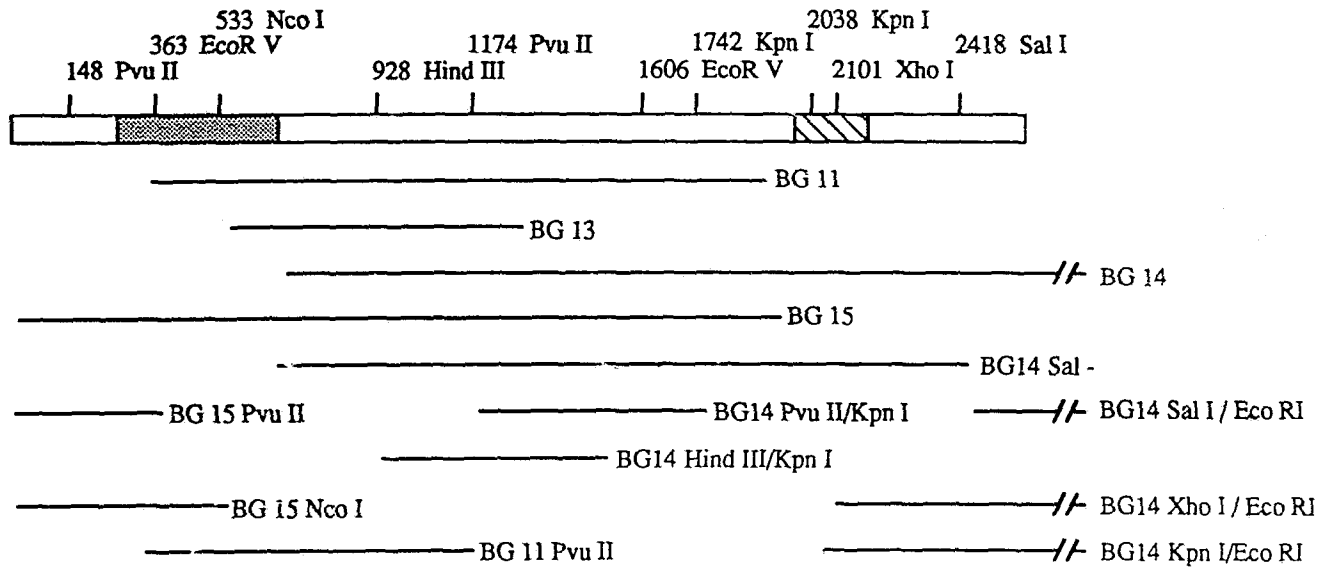


Figure 14: Nucleic acid sequence and predicted amino acid sequence of the sea urchin βG subunit

A potential signal peptide is underlined and the predicted cleavage site is indicated by an arrow. Cysteines are in bold and numbered as described in the text. Potential N-glycosylation sites are indicated with an asterisk. The transmembrane domain is underlined.

```
  1 ctttacccccgcaccagttcccctgatcttaccgaagtttaacactcataatcaccacat 60
 61 taatatattgattttctgatccgtacacgaggaccaggc ATG GCG TGG AAG TGC  114
  1                                          M   A   W   K   C    5

115 CTA CTT GTT CTG ACG ATA GCG TTC AGT GCG GGC CAG CTG TAC AGG  159
  6  L   L   V   L   T   I   A   F   S   A   G   Q   L   Y   R   20

160 ACG TAC GCT CAA ACG TCT TCT AGT GCA TGC AGC GAT GCA AAG ACG  204
 21  T   Y   A   Q   T   S   S   S   A   C   S   D   A   K   T   35
                                     ↑   1
205 TGT GGG GAA TGC ATA TCA TTG GAC TCA TCG TGC GGT TGG TGT ACG  249
 36  C   G   E   C   I   S   L   D   S   S   C   G   W   C   T   50
      2           3                           4           5
250 TTA CTA AAT TAT ACA GAT GAC ACA GGC AAC CCG CAG TGT GAT TTA  294
 51  L   L   N   Y   T   D   D   T   G   N   P   Q   C   D   L   65
              *                                       6
295 GCA AGC AGC TTG AGC CAG AGA GGA TGC TCA CAA ATC GTT GAT CCA  339
 66  A   S   S   L   S   Q   R   G   C   S   Q   I   V   D   P   80
                                     7
340 GAC AGT ACT ATG GTG TTA GGA TGG ATA TCG TTG AGT AAT GCT GGC  384
 81  D   S   T   M   V   L   G   W   I   S   L   S   N   A   G   95

385 AGC GCT CCT CAG GGC CAG GCA GTC CAG GTT CGA CCA CAG CAA GTT  429
 96  S   A   P   Q   G   Q   A   V   Q   V   R   P   Q   Q   V  110

430 GAT CTG AAG CTC AGA AGG GGA AAG CCA GTG GTT ATG ACG CTC CCA  474
111  D   L   K   L   R   R   G   K   P   V   V   M   T   L   P  125

475 GGT CCG ACA GCC GAG GAT TAC CCG GTG GAT CTC TAC TAT GTA ATG  519
126  G   P   T   A   E   D   Y   P   V   D   L   Y   Y   V   M  140

520 GAT CTC TCC AAA TCC ATG GAA GAT GAT CTC TCA AAA CTC ATG GAC  564
141  D   L   S   K   S   M   E   D   D   L   S   K   L   M   D  155

565 CTT GGA GAT ATT CTA GCG AGC GAG ATG AAA AAT ATC ACA AGC AAT  609
156  L   G   D   I   L   A   S   E   M   K   N   I   T   S   N  170
                                         *
610 TTC AGA CTT GGT TTC GGA TCA TTT GTT GAT AAG ACT GTG ATG CCC  654
171  F   R   L   G   F   G   S   F   V   D   K   T   V   M   P  185

655 TAT GTC AGT ACA GTG CCT GAA AAA TTG ATT GCA CCT TGT ACA GGG  699
186  Y   V   S   T   V   P   E   K   L   I   A   P   C   T   G  200
                                             8
700 TGT GAG GCT CCG TAT GGA TTC AAG AAC GTA CTC CCA TTG AAC GAG  744
201  C   E   A   P   Y   G   F   K   N   V   L   P   L   N   E  215
      9
745 AAC ACA AAC TTA TTT TCA GAA ACG GTG ATG AAC CAG AGA GCT TCA  789
216  N   T   N   L   F   S   E   T   V   M   N   Q   R   A   S  230

790 GGA AAC CTA GAT GCA CCT GAG GGA GGC ATG GAT GCT CTC ATG CAA  834
231  G   N   L   D   A   P   E   G   G   M   D   A   L   M   Q  245

835 ATC ACA GTC TGT GGG AAC CAA ATA GGA TGG AGA GAG AAT GCA AGA  879
246  I   T   V   C   G   N   Q   I   G   W   R   E   N   A   R  260
                 10
880 CAT CTT GTG ATC TAC ACC ACC GAT TCT TCT TCC ATT ATG CCG GAG  924
261  H   L   V   I   Y   T   T   D   S   S   S   I   M   P   E  275

925 ACG AAG CTT GGA GGT ATC ATT ACT CCC AAT GAT GGA CAA TGC TAT  969
276  T   K   L   G   G   I   I   T   P   N   D   G   Q   C   Y  290
                                                 11
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```
970 CTG GAC CCT ATT TCA CAG AAC TAT ACT ATG TCA CAT TAC TTG GAC 1014
291  L   D   P   I   S   Q   N   Y   T   M   S   H   Y   L   D  305
                            *

1015 TAC CCA TCC ATC AGA CAT CTC AAT GCT AAG ATG AGA GAG AAC AGT 1059
306  Y   P   S   I   R   H   L   N   A   K   M   R   E   N   S  320

1060 GTC ATC CCC ATC TTT GCC GTC ATC CAG AAG GAG TTT GAG ATC TAC 1104
321  V   I   P   I   F   A   V   I   Q   K   E   F   E   I   Y  335

1105 AAT AAT CTG ACT CAA TAC ATA GAG GGA GCT ACA GCG GGT ATC TTG 1149
336  N   N   L   T   Q   Y   I   E   G   A   T   A   G   I   L  350
         *

1150 GCC CAG GAT TCC AAC AAC ATT GTA CAG CTG GTC AAA GAC AAC TAC 1194
351  A   Q   D   S   N   N   I   V   Q   L   V   K   D   N   Y  365
                                                        *

1195 AGT AAA ATC ACA TCC AGA GTC GAA GTG GTG GAC GAT GCC CCC GAG 1239
366  S   K   I   T   S   R   V   E   V   V   D   D   A   P   E  380

1240 AAC GTG ACC ATC GAC TAC GGA CCC CAT TGT CCT GGA GGA CAG GTC 1284
381  N   V   T   I   D   Y   G   P   H   C   P   G   G   Q   V  395
     *                                   12
1285 ACC CCA GGG TCA CAG GTG TGC GAG GGT CTT CAA CTT GGC GAC ACG 1329
396  T   P   G   S   Q   V   C   E   G   L   Q   L   G   D   T  410
                            13
1330 GTC AAC TTC ACG CTG ACC ATT ACG GCG ACT GGA TGT CCA CCC AAT 1374
411  V   N   F   T   L   T   I   T   A   T   G   C   P   P   N  425
         *                                       14
1375 AAA TAC CAA CAG TTC ACT GTA CGC CCA TTG ATT CAA CGA GAA CTG 1419
426  K   Y   Q   Q   F   T   V   R   P   L   I   Q   R   E   L  440

1420 AAA GTG AAT GTA GAG TTT GCG TGC GAC TGT GAC TGC GAA GCA CAA 1464
441  K   V   N   V   E   F   A   C   D   C   D   C   E   A   Q  455
                                15      16      17
1465 AAG GTT GAG AAT AGT CAG GTA TGC AGT GGA GGA AAT GGC ACC TTG 1509
456  K   V   E   N   S   Q   V   C   S   G   G   N   G   T   L  470
                                18
1510 GAG TGC GGC AGT TGT ATC TGT AAC CCG GGA CAC TAT GGC CGA TAC 1554
471  E   C   G   S   C   I   C   N   P   G   H   Y   G   R   Y  485
         19          20      21
1555 TGT GAG TGC AGC AGT GAC GAT CCT ACG CTA GAA GAC AAT GAC GCA 1599
486  C   E   C   S   S   D   D   P   T   L   E   D   N   D   A  500
     22      23
1600 CCT TGC GAT ATC ACC AAA CAC ATC CAT CGT TGT TCA GGA AGA GGA 1644
501  P   C   D   I   T   K   H   I   H   R   C   S   G   R   G  515
         24                                  25
1645 TCT TGT GTG TGC GGA AAT TGC ATC TGT TTC CCA AGA CCG AAC CCC 1689
516  S   C   V   C   G   N   C   I   C   F   P   R   P   N   P  530
         26      27          28      29
1690 AGT GAA GTT GTT TCT GGC ACT TTC TGT GAA TGT GAT AAC TTC AAC 1734
531  S   E   V   V   S   G   T   F   C   E   C   D   N   F   N  545
                                    30      31
1735 TGT GAT AGG TAC CTA GGA GAG CTT TGT GGT GGT TCT GAT AGA GGG 1779
546  C   D   R   Y   L   G   E   L   C   G   G   S   D   R   G  560
     32                             33
1780 CAG TGT GTA TGC GAC GAG TAC ACG AGG AGA AGT CAG TGC CGG TGC 1824
561  Q   C   V   C   D   E   Y   T   R   R   S   Q   C   R   C  575
         34      35                                  36      37
1825 AGG TCG GGG TAC ACG GGG GAC GCC TGC GAA TGT TCT ACT CGG GTG 1869
576  R   S   G   Y   T   G   D   A   C   E   C   S   T   R   V  590
                                    38      39
1870 GAT ACC TGT ATG ACA GGA GAT ACC ATA TGC AAT GGG GAA GGT GTG 1914
591  D   T   C   M   T   G   D   T   I   C   N   G   E   G   V  605
             40                             41
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1915 TGC ATC TGT GGG GAG TGT AAA TGT AAC GCT GGC TCG TCC TAT AC? 1959
 606  C   I   C   G   E   C   K   C   N   A   G   S   S   Y   R   620
     42      43          44      45
1960 GGA GCA CTA TGC CAA GAC TGT CCG ACT TGT AGC GGT CAG TGT TCT 2004
 621  G   A   L   C   Q   D   C   P   T   C   S   G   Q   C   S   635
                 46          47          48          49
2005 AGG AAC GAG GAG TGC GTC CAG TGC AAG GCT TTT GGT ACC GGA CTG 2049
 636  R   N   E   E   C   V   Q   C   K   A   F   G   T   G   L   650
                     50          51
2050 TCC AAA GCA GAT TGT GAC AAG TGT CCT TTC CCG GTC ATC ATG GTC 2094
 651  S   K   A   D   C   D   K   C   P   F   P   V   I   M   V   665
                     52          53
2095 GAT AAC CTC GAG ATT CCC ACT GGT TCA GAG AGG TGC CTT GCA GAA 2139
 666  D   N   L   E   I   P   T   G   S   E   R   C   L   A   E   680
                                                 54
2140 GAC GAA GAC GAT TGT TCC ATT ATC TTT ACC TAT GCA AAG TCA GCC 2184
 681  D   E   D   D   C   S   I   I   F   T   Y   A   K   S   A   695
                     55
2185 AAC CTG GCC CTC ATC TTA TAT GTG CAG AAA GAA AAA GTA TGC TTT 2229
 696  N   L   A   L   I   L   Y   V   Q   K   E   K   V   C   F   710
                                                     56
2230 GAA CCA GTG GAC ATC ATG CAT GTC ATC ATC GGT ATC GTC GTT GGT 2274
 711  E   P   V   D   I   M   H   V   I   I   G   I   V   V   G   725

2275 ATC ATC ATC GTG GGA TTG GCC CTC CTC CTC GTT TGG AGG TTG CTC 2319
 726  I   I   I   V   G   L   A   L   L   L   V   W   R   L   L   740

2320 GTC TAT GTT CAA GAC AGC AGA GAA TTT GCA TCA TTT GAG AAA GAA 2364
 741  V   Y   V   Q   D   S   R   E   F   A   S   F   E   K   E   755

2365 AGA GCA GGA ACG CAT TGG GGA CAG AAT GAA AAT CCG ATA TAC AAG 2409
 756  R   A   G   T   H   W   G   Q   N   E   N   P   I   Y   K   770

2410 CCA TCA ACG TCG ACA TTC AAG AAC CCC ACA TAC CAG AAA tgagacgg 2456
 771  P   S   T   S   T   F   K   N   P   T   Y   Q   K           783

2457 atttagggtgccatatttttctgttctttttctcctgtgtccttatcttttgttgcatt 2516

2517 caaagacatacaatcctgcatatctgtttgaaagcagcgatttatgaagctctgttcata 2576

2577 cat
```

43% for βL and 30% for βC). The βG subunit contains 56 cysteine residues in the presumptive extracellular domain (Figure 14). All known β integrins with the exception of the β4 (Erle *et al.*, 1991), β7 (reviewed in Yee and Hynes, 1993), β8 (Moyle *et al.*, 1991), and the *Drosophila* βv subunit (Yee and Hynes, 1993) contain these 56 residues. While the distribution of the cysteines is highly conserved in other integrins the βG subunit does not display this conserved pattern (Figure 26). The first seven cysteines that are located within the predicted ligand binding domain are completely conserved as they are in all β subunits examined to date. The location of cysteine 12 (residue 390) does not correspond to that observed in any of the vertebrate integrins, and at the site where cysteine 26 is normally located there is an alanine residue (residue 337) (figure 24).

The predicted ligand binding domain of the βG subunit comprises amino acid residues 133-371 (Figure 14), and this domain is highly conserved among other integrins. The ligand binding domain of the βG subunit bears closest resemblance to the βL subunit (61% similarity). Among the vertebrate subclasses, the βG subunit ligand binding domain most resembles the β1-like integrins (β1, β2, and β3: 50-54% similarity). The cytoplasmic domain of the βG subunit is 39 amino acids long and also bears closest resemblances to that of the β1 integrins.

2.23 Temporal patterns of βG transcript expression

The βG integrin subunit is present in the egg as a maternal transcript of 7.5 kb and gradually increases in prevalence up to a peak at gastrula, before the expression begins to drop through to the pluteus (Figure 15). As described above, the apparent increase in

expression at the cleavage stage of development is likely an artifact of loading and the increase observed in the gastrula stage may also represent an artifact as this lane has the highest concentrations of RNA as estimated from the density of staining on the blot (Figure 15). Like βC, the βG transcript appears to be relatively rare at all stages of development as estimated by the intensity of the signal on northern blots.

2.24 Immunological analysis of βG expression

The rabbit polyclonal antiserum 61CR was raised against the extracellular portion of the βG subunit that encompasses the ligand binding domain to an area outside the plasma membrane (Figure 14). The antiserum was used on immunoblots of membrane extracts separated by both reducing and non-reducing SDS-PAGE (Figure 16). Using extracts made up in reducing sample buffer from gastrula stage embryos 61CR recognizes a single band with an apparent molecular weight of 120 kDa (figure). In samples prepared in non-reducing sample buffer the antiserum recognizes a single band that migrates at an apparent molecular weight of 116 kDa (Figure 16). Preimmune serum isolated from rabbit 61CR before immunization recognizes no proteins on immunoblots prepared from the same lysates described above (Figure 16). Preabsorption of 61CR serum w. the immunogen abolishes recognition of all proteins, as well as the immunogen on immunoblots (Figure 16).

The expression of the βG subunit appears to be developmentally regulated as both the intensity of the bands and the banding patterns observed on immunoblots change as development proceeds. In the egg there are small amounts of the βG protein and it

Figure 15: Northern blot analysis of βG

Northern blots were performed on 10μg of total RNA isolated from egg (E), cleavage (C), blastula (B), gastrula (G), and pluteus (P) stage embryos.

A. Methylene blue stained membrane showing localization of 28S and 18S ribosomal RNAs.

B. The same blot as in A hybridized with a probe made from the SpβG PCR product. A single band of 7.5 Kb is visible at all stages. βG expression peaks in the cleavage and blastula stages. The size of the βG transcript was estimated from the rRNAs.

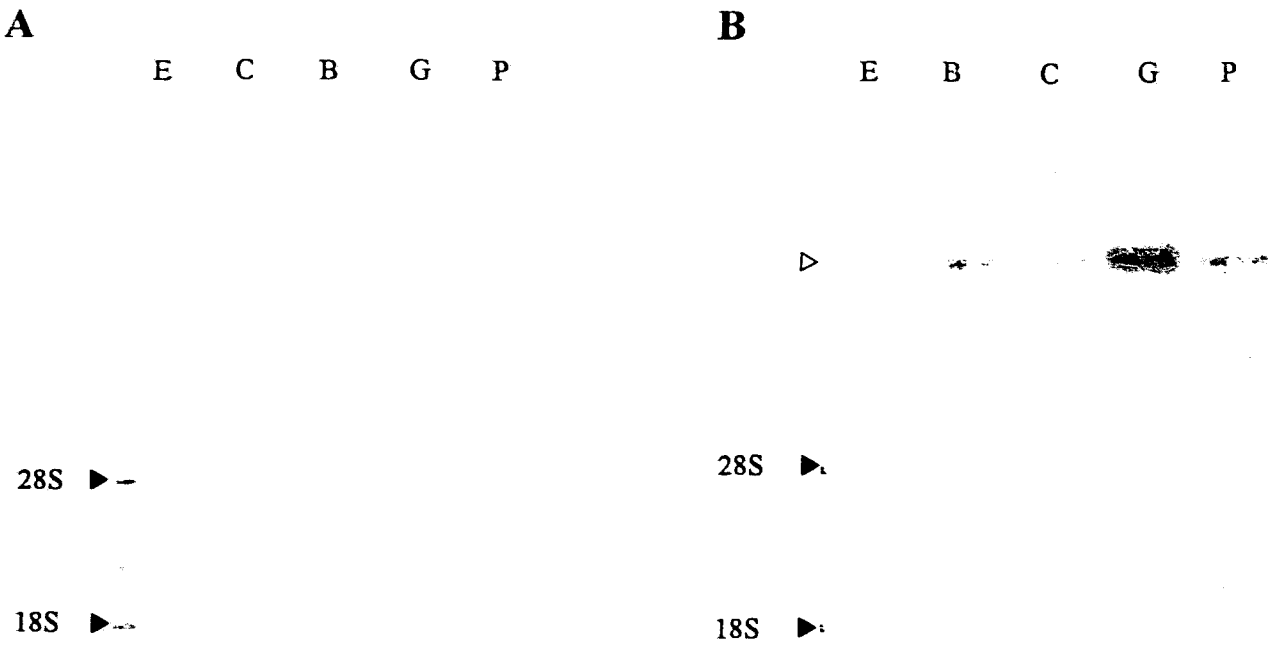

A
E C B G P
28S
18S
B
E B C G P
28S
18S

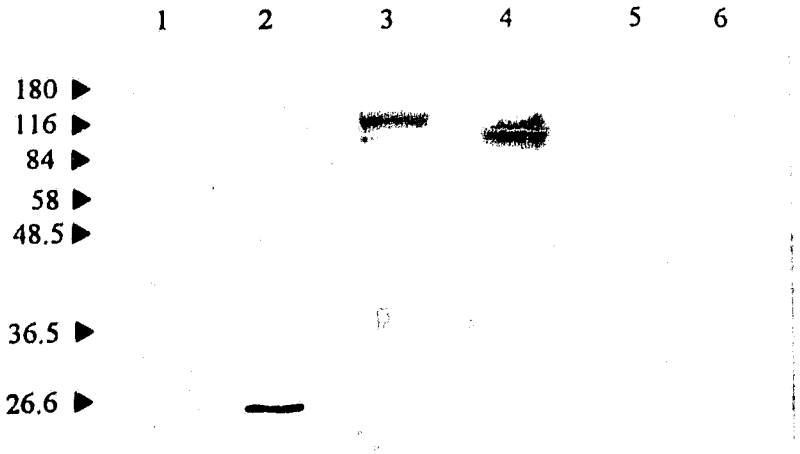
Figure 16: Analysis of the βG protein by western blotting using the 61CR antiserum

A. Midgastrula membrane preparations were seperated by SDS-PAGE and western blots performed with preimmune serum (lane 1), immune serum on samples run in the presence of reducing sample buffer (lane 3), nonreducing sample buffer (lane 4). Lane 2 represents a western blot of the expressed protein to which the antiserum was raised probed with immune serum. Preabsorption with the expressed protein abolishes recognition of midgastrula samples (lane 5), and the expressed protein (lane 6).

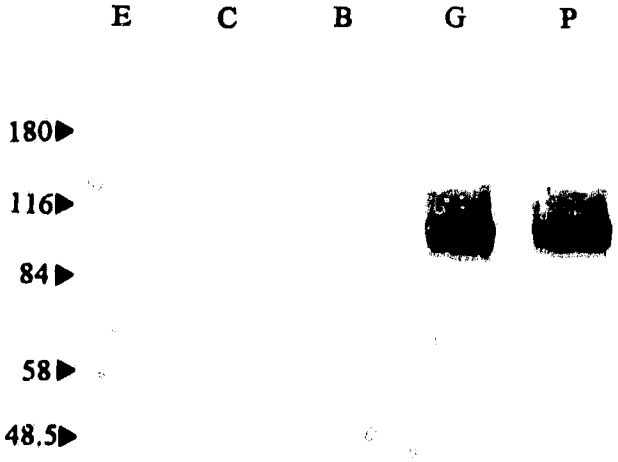
B. Western blots were performed on membrane preparations run under nonreducing conditions made from eggs (E), cleavage (C), blastula (B), gastrula (G), and pluteus (P) stage embryos. A number of bands centered at an apparent molecular weight of 116 kDa is evident at all stages.

C. Same samples as in B run under reducing conditions. A triplet of bands is clear in all samples. In eggs the upper two bands dominate, while in blastula the lower bands dominate. In the later stages the middle band appears to be the most abundant.

A



B



C

appears as three distinct bands (Figure 16). During cleavage and blastula stages of development, the relative distribution of these bands is altered. By the gastrula stage of development there is a dramatic increase in expression of the molecule that decreases into the pluteus stage of development. At these later stages of development a single band dominates the expression pattern of βG.

2.25 *In situ* localization of the βG subunit

cRNA probes were synthesized using the βG14 clone as a template and used for *in situ* localizations. In all situations embryos hybridized with the probe corresponding to the sense strand took up no stain and were a pale tan or very pale blue colour. Embryos at all stages and in all tissues stain a light blue colour with the antisense probe and perhaps this reflects a constitutive level of expression. Embryos before the PMC blastula stage did not reveal any specific localization of the transcript, presumably due to the low levels of the transcript present during these stages (Figure 15). Localization at this time can be difficult as the large quantities of yolk in early embryos cause high background. At the PMC blastula stage the βG subunit transcripts become detectable and are localized to the area where the PMCs are ingressing (Figure 17). The PMCs stain strongly for βG as they move into the blastocoel but then cease to hybridize probe (Figure 17). While the results are variable, the pigment cells appear positive for the presence of the βG transcript as they detach from the archenteron at the early stages of gastrulation (Figure 17). The βG subunit is found at low levels in the archenteron from the initial stages of invagination up until the pluteus stage of development. Once the archenteron has started to advance

Figure 17: *In situ* analysis of early stage embryos with the βG subunit.

A. Primary mesenchyme blastula hybridized with sense βG probe.

B. Primary mesenchyme blastula hybridized with antisense βG probe. βG expression is detected in all cells of the embryo including the PMCs (arrow).

C. Early gastrula hybridized with sense βG probe.

D. Early gastrula hybridized with antisense βG probe. High levels of expression are detected in the archenteron and presumptive pigment cells (a), PMCs (p). Levels of expression in the lateral ectoderm are decreasing.

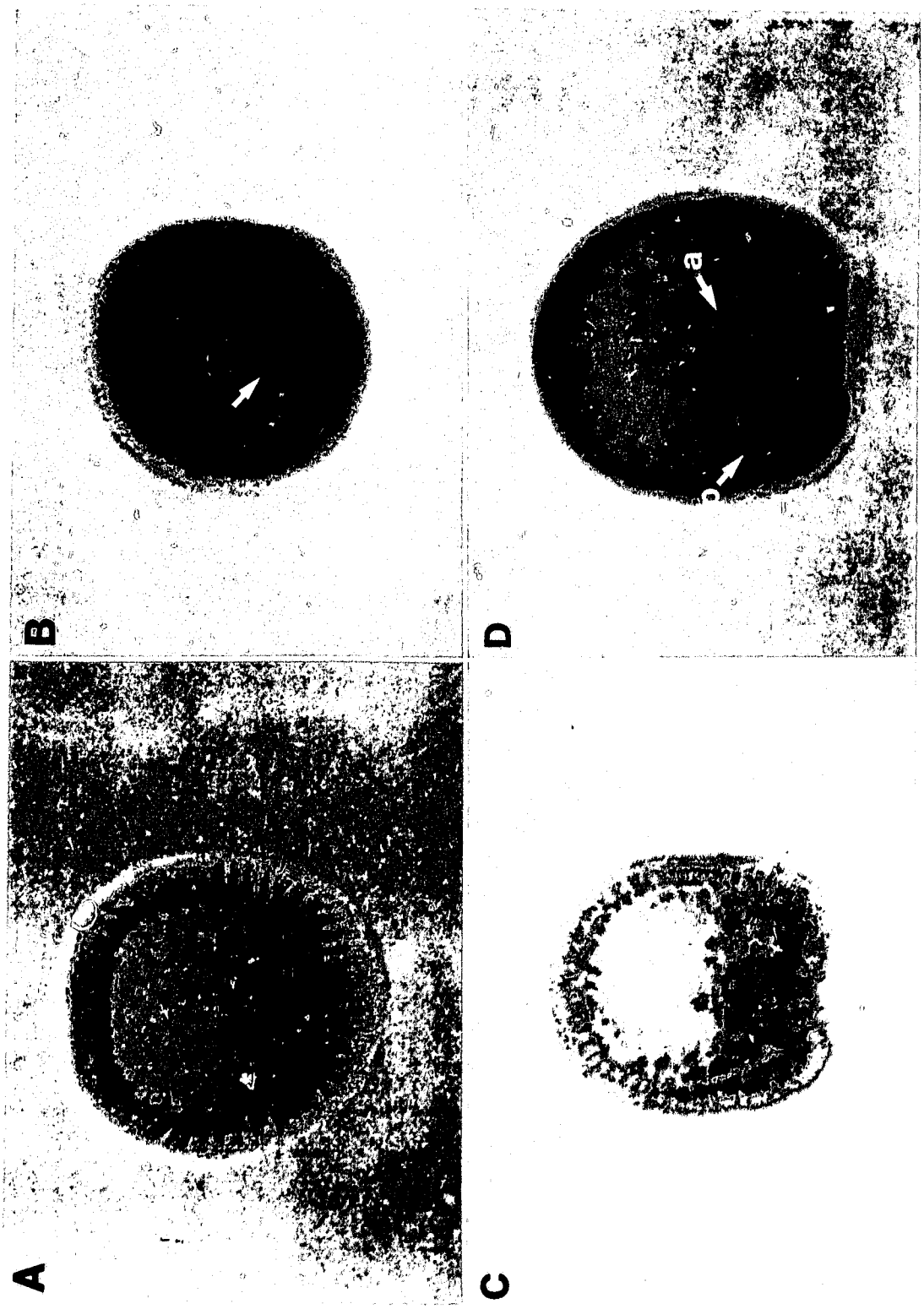

A
B
C
D

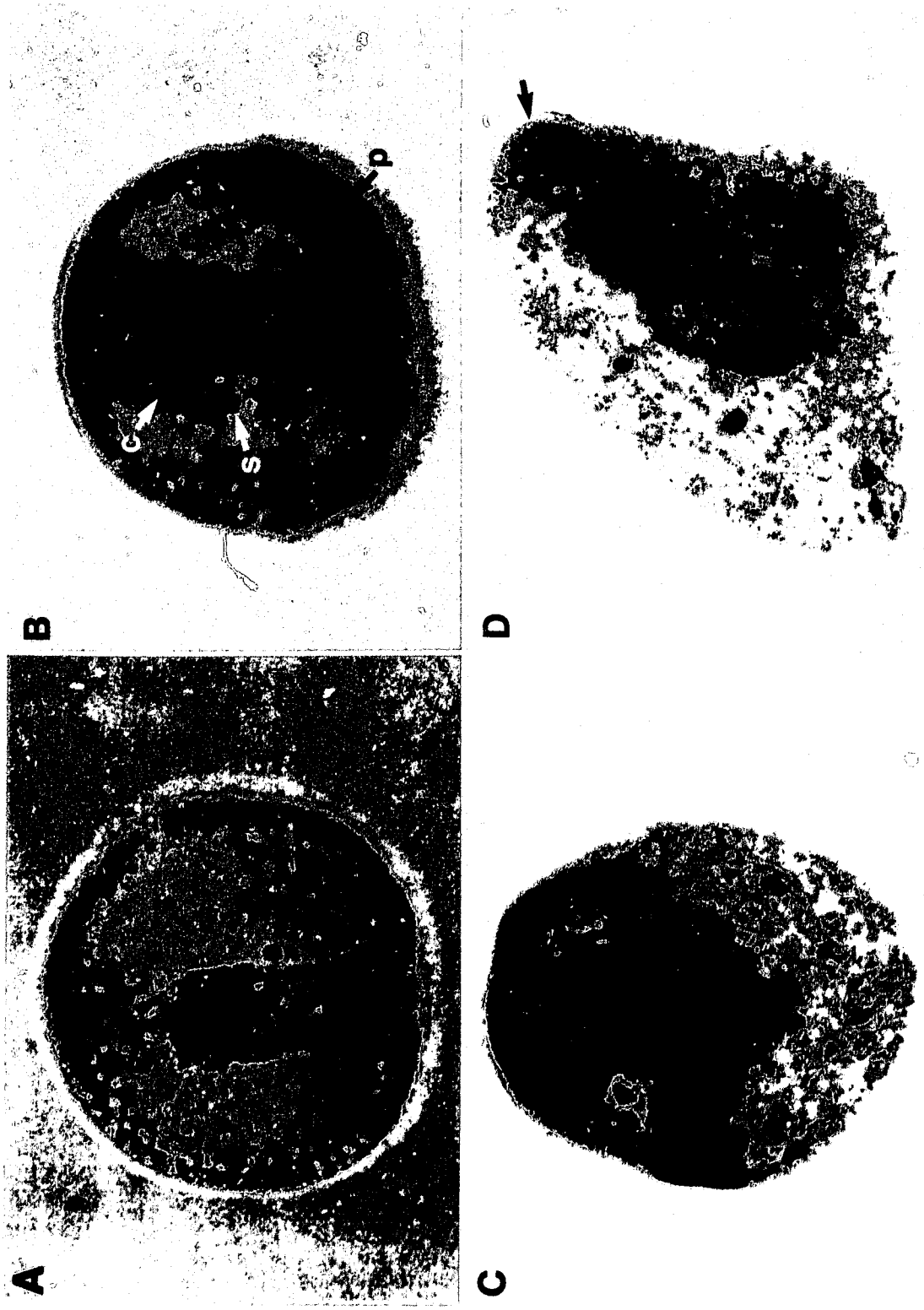
Figure 18: *In situ* analysis of late stage embryos with the βG subunit.

A. Late gastrula hybridized with sense βG probe.

B. Late gastrula hybridized with antisense βG probe. High levels of expression are found in the archenteron, coelomic pouches (c), and the vegetal ectoderm around the lateral margins of the blastopore. SMCs that have detatched from the archenteron no longer express βG (s). PMCs express βG until late gastrulation (p).

C. Early Prism hybridized with antisense βG probe (dorsal view). There are high levels of βG expression in the archenteron and animal cap, as well as increasing levels in the oral face.

D. Prism hybridized with antisense βG probe (lateral view). βG expression is high in pigment cells (p). The archenteron and oral face also express βG. Higher levels of expression are localized to the future sites of the oral arms (arrow).


A
B
C
D
p
c
s

across the blastocoel the transcripts can no longer be localized to the PMCs or pigment cells but it is still present in the archenteron (Figure 18). The βG transcript can be found in the SMCs as long as they are associated with the archenteron, however once they detach from the tip of the archenteron and migrate into the blastocoel the expression of βG is lost (Figure 18). As the embryo begins to acquire the distinctive prism shape the βG transcript can be found to be expressed at high levels in the archenteron (Figure 18). Pigment cells express high levels of βG while they are still in the blastocoel (Figure 18). The only other site of ectodermal staining is at the site of the animal cap and perhaps in the oral face, although it is difficult to distinguish if this is background staining at this location (Figure 18). The level of expression in the archenteron at this time appears to be decreasing although it remains detectable at all times (Figure 18). At the pluteus stage the archenteron stains lightly and the pigment cells in the ectoderm still stain strongly.

2.3 The βL subunit

2.31 Primary sequence of the βL subunit

The βL subunit was isolated as two overlapping cDNAs (Figure 19) that encompass a 2402 bp open reading frame and approximately 1 Kb of untranslated sequence (Figures 20). The 2402 bp open reading frame of βL predicts a protein with a molecular weight of 88 kDa. At the 5' end of the deduced open reading frame a sequence rich in hydrophobic residues extends from the first usable ATG initiation codon to the predicted signal peptide cleavage site at the histidine residue at position 31 (Figure 20, von Heijne, 1986). The extracellular domain contains seven predicted sites for N-

Figure 19: Map of the sea urchin βL subunit cDNAs

A schematic of the predicted protein is aligned with cDNAs obtained from a midgastrula stage cDNA library (BL 1, BL 2). For the protein sequence the PCR fragment is indicated by a stippled region and the transmembrane domain by a hatched region. Subclones are named after the cDNA from which they are derived including the restriction enzyme(s) used to isolate that frgament. Restriction enzyme sites used to generate subclones are indicated. All subclones were sequenced from either end using T7 and T3 primers. Fragments BL 1 primer and BL 2 primer were sequenced using primer walking.

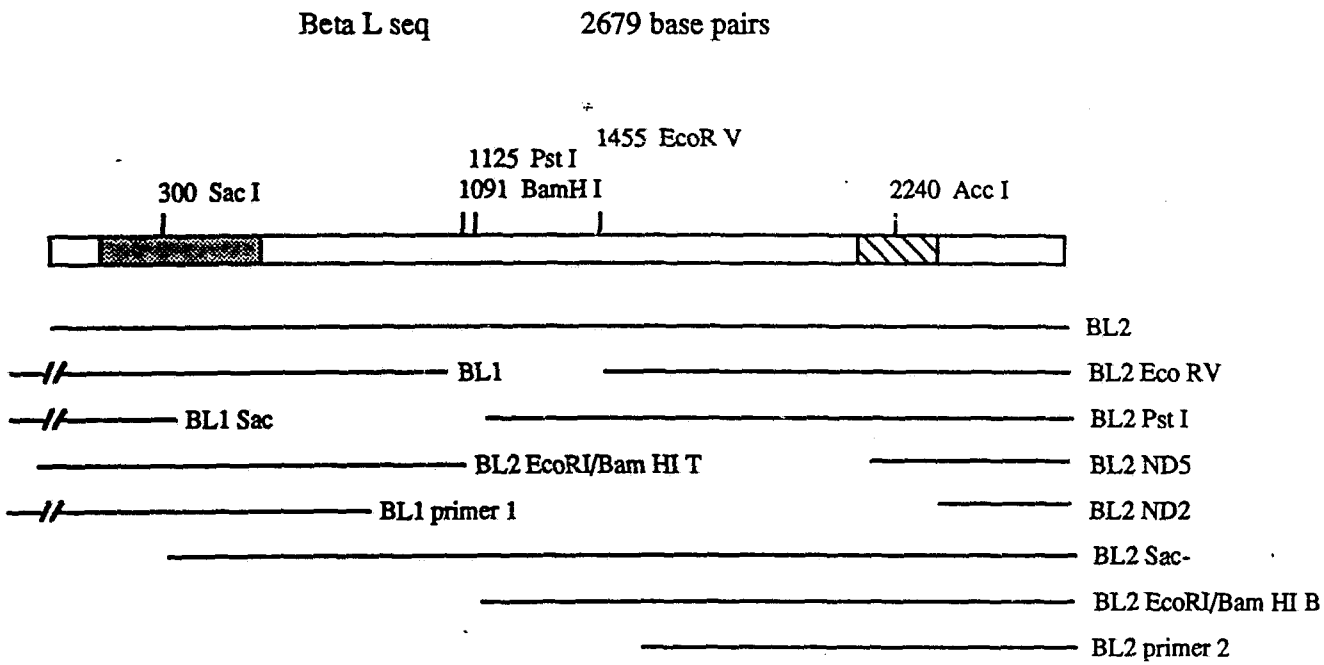

Beta L seq
2679 base pairs
1455 EcoR V
1125 Pst I
1091 BamH I
300 Sac I
2240 Acc I
BL2
BL1
BL2 Eco RV
BL1 Sac
BL2 Pst I
BL2 EcoRI/Bam HI T
BL2 ND5
BL1 primer 1
BL2 ND2
BL2 Sac-
BL2 EcoRI/Bam HI B
BL2 primer 2

Figure 20: Nucleic acid sequence and predicted amino acid sequence of the sea urchin βL subunit.

The potential signal peptide is underlined and the predicted cleavage site is indicated by an arrow. Cysteines are in bold and numbered as described in the text. Potential N-glycosylation sites are indicated with an asterisk. The transmembrane domain is underlined.

```
   1 tttttatatataaattaacaacaacgactggcattgttattcacctggcaacaccctggt  60
  61 atctttcttctggtccggatagaaaggattgcgtttaaccgcggtaaatatcgacttgtt 120
 121 ttgtgagaagtcgtttcgcagacgacggcggagagatatattcccgccaaactacaagga 180
 181 gagataaatcattgaag ATG CCG TCA GTC AGA TTA CCA CAT AGG ACA      227
   1                    M   P   S   V   R   L   P   H   R   T       10

 228 ACC CGT CCT GGA AGT GTA GTC GTT TTC TTT CTC ACA TTC GTC CTA  272
  11  T   R   P   G   S   V   V   V   F   F   L   T   F   V   L   25

 273 GCA GTT TTC ACA GTA CAT GCA AAT GAA GAG CTC AGC TGC GAT CTG  317
  26  A   V   F   T   V   H   A   N   E   E   L   S   C   D   L   40
                                  ↑                   1
 318 TCA AGA GCA CAG AAC TGT GGA GAA TGT ATA TCT GTG AAT CCA GAG  362
  41  S   R   A   Q   N   C   G   E   C   I   S   V   N   P   E   55
                          2           3
 363 TGT ACA TGG TGT AAG GAA GAT GTA TTC GAG GGG CGA AGA TGC GAT  407
  56  C   T   W   C   K   E   D   V   F   E   G   R   R   C   D   70
      4           5                                       6
 408 CTT GAG ATC CTC TTG CAA GAA GCT GGA TGC GGC AAC ATC ACC AAT  452
  71  L   E   I   L   L   Q   E   A   G   C   G   N   I   T   N   85
                                          7       *
 453 CCT CTT CCG TCT GCC GTA CCC ATA GAA GAC AAA CCA CTG AGT GAG  497
  86  P   L   P   S   A   V   P   I   E   D   K   P   L   S   E  100

 498 GCC AAT GCT GAT CTG GAT GCA ATC GTT CAA GTC AAG CCT CAG ATG  542
 101  A   N   A   D   L   D   A   I   V   Q   V   K   P   Q   M  115

 543 ATG AGG ATC AAA GTA CGA CCG AGG GAG CCA ATC AAC ATA AAG TTG  587
 116  M   R   I   K   V   R   P   R   E   P   I   N   I   K   L  130

 588 TAC GTG CGC CAA GCC GAA GAC TAC CCT GTC GAT CTC TAC TAC GCT  632
 131  Y   V   R   Q   A   E   D   Y   P   V   D   L   Y   Y   A  145

 633 ATG GAT CTC AGT CAC TCC ATG AAA GAC GAT CTG GAA AAC CTC AAG  677
 146  M   D   L   S   H   S   M   K   D   D   L   E   N   L   K  160

 678 GGT CTC GGA ACA ACC TTA TCG GAA GAG TTG AAT AGC ATT ACC CGT  722
 161  G   L   G   T   T   L   S   E   E   L   N   S   I   T   R  175

 723 GAC TTT CGG CTT GGG TTC GGC TCC TTT GTG GAT AAG ACA GTT CTA  767
 176  D   F   R   L   G   F   G   S   F   V   D   K   T   V   L  190

 768 CCT TAC GTA AGC ACT GTT CCC GCC CAA CTG ATT TCT CCA TGT ACA  812
 191  P   Y   V   S   T   V   P   A   Q   L   I   S   P   C   T  205
                                                      8
 813 GGA TGC GCT AGT CCA CAC GGA TTC CAC AAT GCA CTA CCT CTA AAT  857
 206  G   C   A   S   P   H   G   F   H   N   A   L   P   L   N  220
          9
 858 CAA GAT CCA TCG CTC TTT GCT AAC CGT ATC ACA AAC ACC ACA GTG  902
 221  Q   D   P   S   L   F   A   N   R   I   T   N   T   T   V  235
                                              *
 903 TCT GGT AAC TTG GAT ACT CCT GAG GGT GGG TTC AGC GCT TTG ATG  947
 236  S   G   N   L   D   T   P   E   G   G   F   S   A   L   M  250

 948 CAG ATT GCT GTT TGT GGG GAG GTC ATC GGT TGG AGA CCC AAA GCT  992
 251  Q   I   A   V   C   G   E   V   I   G   W   R   P   K   A  265
                      10
 993 CGC CAT CTG GTA ATC TTT ACA ACA GAC GCT TCA TTC CAT TTT GCG 1037
 266  R   H   L   V   I   F   T   T   D   A   S   F   H   F   A  280

1038 GGA GAC GGA AGA CTT GGA GGC ATT GTG GAA CCC AAT GAC GGT CAG 1082
 281  G   D   G   R   L   G   G   I   V   E   P   N   D   G   Q  295
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1083 TGT CAC ATG GAT CCT AAC ACA AAC ATG TAC GAC TTT AGT ACC CTG 1127
 296  C   H   M   D   P   N   T   N   M   Y   D   F   S   T   L  310
      11
1128 CAG GAT TAT CCC TCT ATC GGT CAT CTC AGC GCC AAG CTA CGA GAA 1172
 311  Q   D   Y   P   S   I   G   H   L   S   A   K   L   R   E  325

1173 AAC AAC GTC ATT CCT ATT TTC GCT GTT ACC AGG GAC CAA ACG CCC 1217
 326  N   N   V   I   P   I   F   A   V   T   R   D   Q   T   P  340

1218 CTG TAC ATG AGT CTG GAG AAG GAC ATC GAG GGA GCG ACA GTC GGG 1262
 341  L   Y   M   S   L   E   K   D   I   E   G   A   T   V   G  355

1263 ACG CTT GAC GAA GAC TCT GGA AAC GTG GTG CAG CTC ATT CGT TCA 1307
 356  T   L   D   E   D   S   G   N   V   V   Q   L   I   R   S  370

1308 AAT TGT GAC CGT ATC ACA TCG CAA GTC CGA CTG ACC TCA ACA GCT 1352
 371  N   C   D   R   I   T   S   Q   V   R   L   T   S   T   A  385
          12
1353 CCC GAT GAC GTC ACA CTC TCT TAC CGA GCG AAC TGT AAA GAT CAG 1397
 386  P   D   D   V   T   L   S   Y   R   A   N   C   K   D   Q  400
                                                  13
1398 ACA TAC CAA GAC ACG AAC GAG TGT TCG GGA CTC AGT CTA GGG GAC 1442
 401  T   Y   Q   D   T   N   E   C   S   G   L   S   L   G   D  415
                              14
1443 ACA GTA TCC TTT GAT ATC ACC CTC ACG GCC GAG AGG TGT GTC GAA 1487
 416  T   V   S   F   D   I   T   L   T   A   E   R   C   V   E  430
                                                      15
1488 GGC GGC ATG ACG AGT TTT AAC ATT GGA CCA GTA GGA TTC AAT GAA 1532
 431  G   G   M   T   S   F   N   I   G   P   V   G   F   N   E  445

1533 GAA CTT CAG ATT GAG CTA GAA GTG ACG TGT GAA TGC GAT TGT CAG 1577
 446  E   L   Q   I   E   L   E   V   T   C   E   C   D   C   Q  460
                                          16      17      18
1578 GGA TTA GAG GAA GCC AAC AGC ACT GTT TGC AGC AGT GGT AAC GGG 1622
 461  G   L   E   E   A   N   S   T   V   C   S   S   G   N   G  475
                          *                   19                *
1623 ACG TTG GTG TGC GGT GAG TGT GAT TGC AAC CCT GGA CGT TAC GGA 1667
 476  T   L   V   C   G   E   C   D   C   N   P   G   R   Y   G  490
                  20          21      22
1668 GTC AAG TGT GAA TGC AGC GGT AAT GAA ATC AAC ATG GAG AGC ACC 1712
 491  V   K   C   E   C   S   G   N   E   I   N   M   E   S   T  505
              23      24
1713 GAT CCA TCC CCA TGC AGA ACG GAT AAC ACA ACA AGA ACT TGT TCT 1757
 506  D   P   S   P   C   R   T   D   N   T   T   R   T   C   S  520
                      25                  *                26
1758 GGA CGT GGA GAA TGC ATC TGT GGG AAA TGT GTC TGT GAC AAC ACT 1802
 521  G   R   G   E   C   I   C   G   K   C   V   C   D   N   T  535
                      27      28          29      30
1803 GGG AAC CCG GGT GAG GTT ATC TCT GGA CAA TTC TGT GAA TGT GAT 1847
 536  G   N   P   G   E   V   I   S   G   Q   F   C   E   C   D  550
                                                  31      32
1848 AAC TTC AAC TGT CCC TAC AGC CGA GGA TTG CGT TGT GGA GGG CCT 1892
551   N   F   N   C   P   Y   S   R   G   L   R   C   G   G   P  565
              33                                  34
1893 GAC CAA GGA ATG TGT GTT TGT GAC GTA GCT ACA AGA CAA CCT AAG 1937
 566  D   Q   G   M   C   V   C   D   V   A   T   R   Q   P   K  580
                      35      36
1938 TGC CAA TGC AAC CCG GGC TTC GAG GGA GAT AGT TGT GAC TGT CCA 1982
 581  C   Q   C   N   P   G   F   E   G   D   S   C   D   C   P  595
      37      38                                      39      40
1983 ACA CGT TTT GAC ATG TGT GCG GCC AGC AAT GGA TTG GAA TGT AAC 2027
 596  T   R   F   D   M   C   A   A   S   N   G   L   E   C   N  610
                          41                              42
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2028 GCG CAC GGA ACC TGT GTG TGC GGC TGC CTG CGA GTG TTT GCG GAC 2072
 611  A   H   G   T   C   V   C   G   C   L   R   V   F   A   D  625
                     43       44       45
2073 TCA CAG TTC CAG GGA AAG ACG TGT GAG AAA TGC CCC ACA TGT GCA 2117
 626  S   Q   F   Q   G   K   T   C   E   K   C   P   T   C   A  640
                                 46           47           48
2118 TTT GGA ATC TGT CAC ATA CAC CGA GAT TGT GTC GAG TGT ACA GTG 2162
 641  F   G   I   C   H   I   H   R   D   C   V   E   C   T   V  655
                 49                       50           51
2163 TTT GGA ACA GGC AGG CTA ACA CCC GAA CAG TGC GAT ATG TGC ACT 2207
 656  F   G   T   G   R   L   T   P   E   Q   C   D   M   C   T  670
                                             52           53
2208 GTC AAC ATT ATC AAC GTT ACA AGT ATT GAC GAG TAT ACG GAA GAC 2252
 671  V   N   I   I   N   V   T   S   I   D   E   Y   T   E   D  685
             *
2253 AAC CCC AAA TGT AAC TTT CCA CTG AGC GAC GAC TGC ACT TTT CAG 2297
 686  N   P   K   C   N   F   P   L   S   D   D   C   T   F   Q  700
                 54                               55
2298 TTT GTT GTT GTC TCG GAG AAC GAA ACA GTC ACT GTA TAT GTA GAG 2342
 701  F   V   V   V   S   E   N   E   T   V   T   V   Y   V   E  715
                             *
2343 GGG AGA GAA ACA TGC ATC GAA CCG GTG GGC AAG CCA ACG CTG CTC 2387
 716  G   R   E   T   C   I   E   P   V   G   K   P   T   L   L  730
                         56
2388 GGT GGA AGA ATC AGA TGG ATC GTC ATC GGA ATC ATC CTG GGA ATC 2432
 731  G   G   R   I   R   W   I   V   I   G   I   I   L   G   I  745

2433 GTG CTG ATC GGG ATG ATC CTG GCT CGC GCC TGG AGG TTA TAC ACC 2477
 746  V   L   I   G   M   I   L   A   R   A   W   R   L   Y   T  760

2478 TAC GTC CAA GAT AAG AGA GAG TAT GCT CAG TGG GAG AAC GAC TGT 2522
 761  Y   V   Q   D   K   R   E   Y   A   Q   W   E   N   D   C  775

2523 AAG AAA GCC CAA TGG GAT CAG AGT GAT AAC CCC ATC TAC AAG TCA 2567
 776  K   K   A   Q   W   D   Q   S   D   N   P   I   Y   K   S  790

2568 TCG ACA ACG ACA TTC AAG AAT CCA ACA TAT  CCC AAA taacgtacact 2616
 791  S   T   T   T   F   K   N   P   T   Y   G   K                800

2617 cctatgtctgcaatgggataccaatctccttatttatattactggctataaagaagatag 2676

2677 cgtac
```

glycosylation Figure 20). The presumptive transmembrane domain is 25 residues long (and encompasses amino acids 729-753 (Figure 20). The cytoplasmic portion of the molecule is 43 amino acids long.

2.32 Comparison of the βL subunit to other β integrins

The βL subunit closely resembles the βG subunit (Table 2, 43% identity at the amino acid level) although at the nucleic acid level it is clear they are derived from separate genes. In comparison the βL subunit shares approximately 36% identity with the human β subunits (Table 2). Like the βG subunit, the βL subunit has the closest identity to the β1 like integrins (33-40% identity).

Like most of the β integrin subunits, the βL subunit has 56 cysteine residues in the predicted extracellular domain (Figure 20). Similar to the βG subunit the cysteine located at position 12 (residue 372) is out of register with those found in the vertebrate β integrins and also does not align with cysteine 12 in the βG subunit (Figure 26). Unlike βG in which the cysteine normally located at position 26 is missing, in βL the cysteine normally located at position 45 (residue 633) is missing, allowing for an equal number of paired cysteine residues in the extracellular domain (Figure 26).

The ligand binding domain extends from amino acid 138 to residue 383 and bears closest resemblance to the sea urchin βG subunit (61%). Like βG, the βL ligand binding domain is highly conserved and is approximately 50% similar to that of the β1 integrins. The predicted cytoplasmic domain resembles that found in the β1 integrins, and is very

similar to the βG subunit. This is particularly apparent in the last 23 amino acids in which 16 are conserved between the two molecules (Figure 26).

2.33 Temporal expression of the βL subunit

The βL transcript is detected as a single 7.5 Kb transcript on Northern blots. The gene is expressed as a maternal transcript and at low levels up until gastrulation (Figure 21). At this time there is an increase in the prevalence of the transcript that continues through the prism and pluteus stages. In the pluteus stage the transcript appears to be slightly larger than that detected at earlier stages (Figure 21). This is unlikely to be an artifact of loading as the same transfer was probed and stripped for all Northerns and no other sea urchin β subunit shows this apparent increase in size. The intensity of the bands on the βL Northern is much greater than that observed on the Northerns probed with the other sea urchin β subunits suggesting that this transcript is more prevalent than the others.

2.34 Immunological analysis of the βL subunit

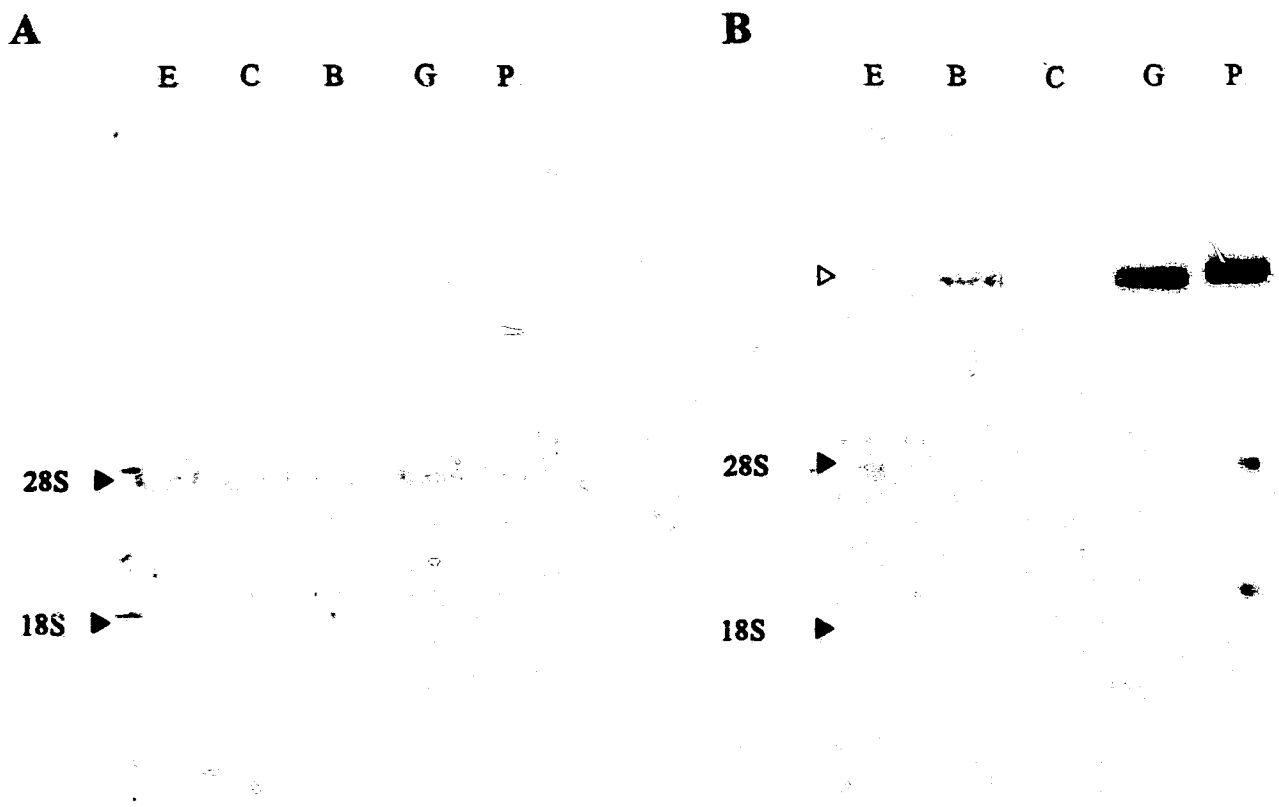
A rabbit polyclonal antiserum was raised against a fragment of the βL subunit that encompasses the extracellular portion from the ligand binding site up to the area outside the cytoplasmic membrane (Figure 22). The 73CR antiserum recognizes a single band with an apparent molecular weight of 120 kDa when used on immunoblots made from proteins separated by nonreducing SDS-PAGE. Upon reduction the apparent molecular mass of the βL subunit increases to an apparent molecular weight of 120 kDa (Figure 22).

Figure 21: Northern blot analysis of βL

Northern blots were performed on 10μg of total RNA isolated from egg (E), cleavage (C), blastula (B), gastrula (G), and pluteus (P) stage embryos.

A. Methylene blue stained membrane showing localization of 28S and 18S ribosomal RNAs.

B. The same blot as in A hybridized with a probe made from the βL PCR product. A single band of 7.5 Kb is visible at all stages. βL expression peaks in the cleavage and blastula stages. The size of the βL transcript was estimated from the rRNAs.


A
E C B G P
28S
18S
B
E B C G P
28S
18S

archenteron stain positively for βL (Figure 23). During the time that the archenteron Preabsorption with the expressed fragment abolishes all recognition (Figure 22), and the serum isolated before immunization recognizes no proteins on similar blots (Figure 22). On reducing blots the antiserum recognizes contaminants on the blots that appear as streaks in all lanes that are run with reducing sample buffer. Preabsorbtion with the expressed fragment removes all traces of this recognition suggesting that it is specific. It remains unknown what this contaminant is as it remains even after all solutions are 0.22 μm filtered.

The β L subunit displays developmentally regulated expression. The protein is barely detectable in early stages, however at the time when gastrulation is initiated there is an increase in the amount of the βL subunit detected. This is maintained into the pluteus stage of development (Figure 22). The 73CR serum does not display a strong recognition on immunoblots and this is due to the restricted boosting schedule the rabbit has been subjected to. This serum also weakly recognizes many epitopes on western blots and this is likely a reflection of low titre.

2.35 *In situ* localization of the βL transcript

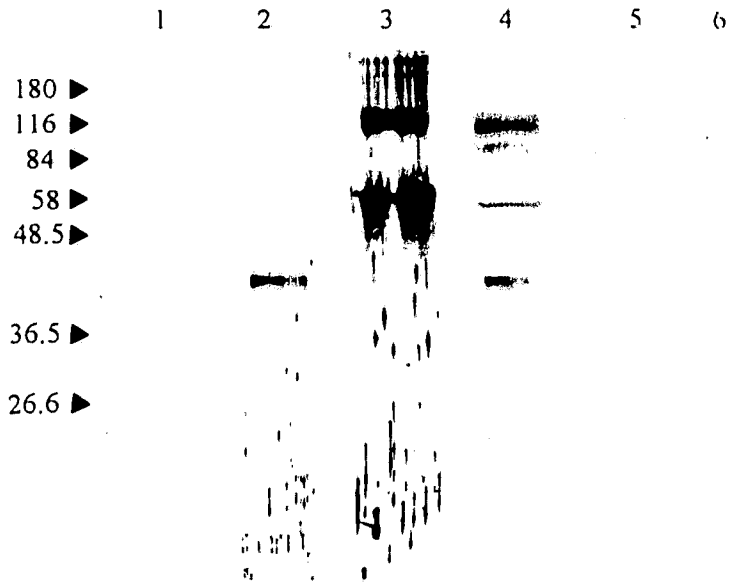
Sense and antisense digoxigenin labelled cRNA probes were made for *in situ* analysis using the βL46 Eco RI/Bam HI subclone. The βL transcript is not detectable by *in situ* hybridization before the early gastrula stage of development. At the time that the PMCs are ingressing βL transcripts become detectable in the vegetal plate (Figure 23).

Figure 22: *Analysis of the βL protein by western blotting using the 73CR antiserum*

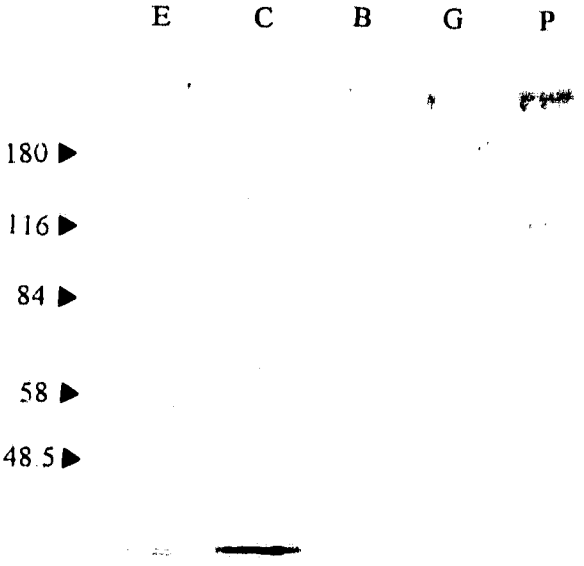
A. Midgastrula membrane preparations were separated by SDS-PAGE and western blots performed with preimmune serum (lane 1), immune serum on samples run in the presence of reducing sample buffer (lane 3), nonreducing sample buffer (lane 4). Bands in lane 4 migrating at an apparent molecular weight of 45 kDa and 56 kDa are assumed to be degradation of the βL subunit in this sample. Lane 2 represents a western blot of the expressed protein to which the antiserum was raised probed with immune serum. Preabsorption with the expressed protein abolishes recognition of midgastrula samples (lane 5), and the expressed protein (lane 6).

B. Western blots were performed on membrane preparations run under nonreducing conditions made from eggs (E), cleavage (C), blastula (B), gastrula (G), and pluteus (P) stage embryos. A doublet is recognized that migrates near an apparent molecular weight of 116 kDa in the gastrula (G) and prism (P) lanes.

A



B



At this time it would appear that only five to seven cells are expressing the βL gene. As the vegetal plate buckles to form the archenteron the cells that will form the tip of the extends the cells at the tip continue to express the βL transcript (Figure 24). During this time there is a varying amount of expression observed in cells that are located within the blastocoel. These cells appear to be mostly at the vegetal pole and are intermixed with, or perhaps are a subset of, the PMCs (Figure 24). In some embryos the ring of PMCs appear to express the βL transcript and remain positive until they become syncytial and form spicules. As the SMCs send out filopodial extensions that contact the ECM of the apical cap they begin to strongly express the βL gene (Figure 24). Some of these cells retain expression of the βL subunit as they migrate vegetally close to the archenteron. Other SMCs that remain in the animal hemisphere lose expression of βL quickly. In the prism and pluteus stages of development the PMCs and SMCs have down regulated the expression of the βL subunit. Numerous cells that line the ectodermal surface of the blastocoel express the βL subunit at high levels in the late gastrula and later stages (Figure 24). By morphological criteria these cells appear to be a heterogeneous population as some of the cells contain very large nuclei.

Figure 23: *In situ* analysis of early stage embryos with the βL subunit

A. Early gastrula hybridized with antisense βL probe. A small number of cells in the vegetal plate express βL (arrow).

B. Early gastrula hybridized with sense βL probe.

C. Mid gastrula hybridized with antisense βL probe. The cells at the tip of the extending archenteron express βL (arrow).

D. Mid gastrula hybridized with sense βL probe.

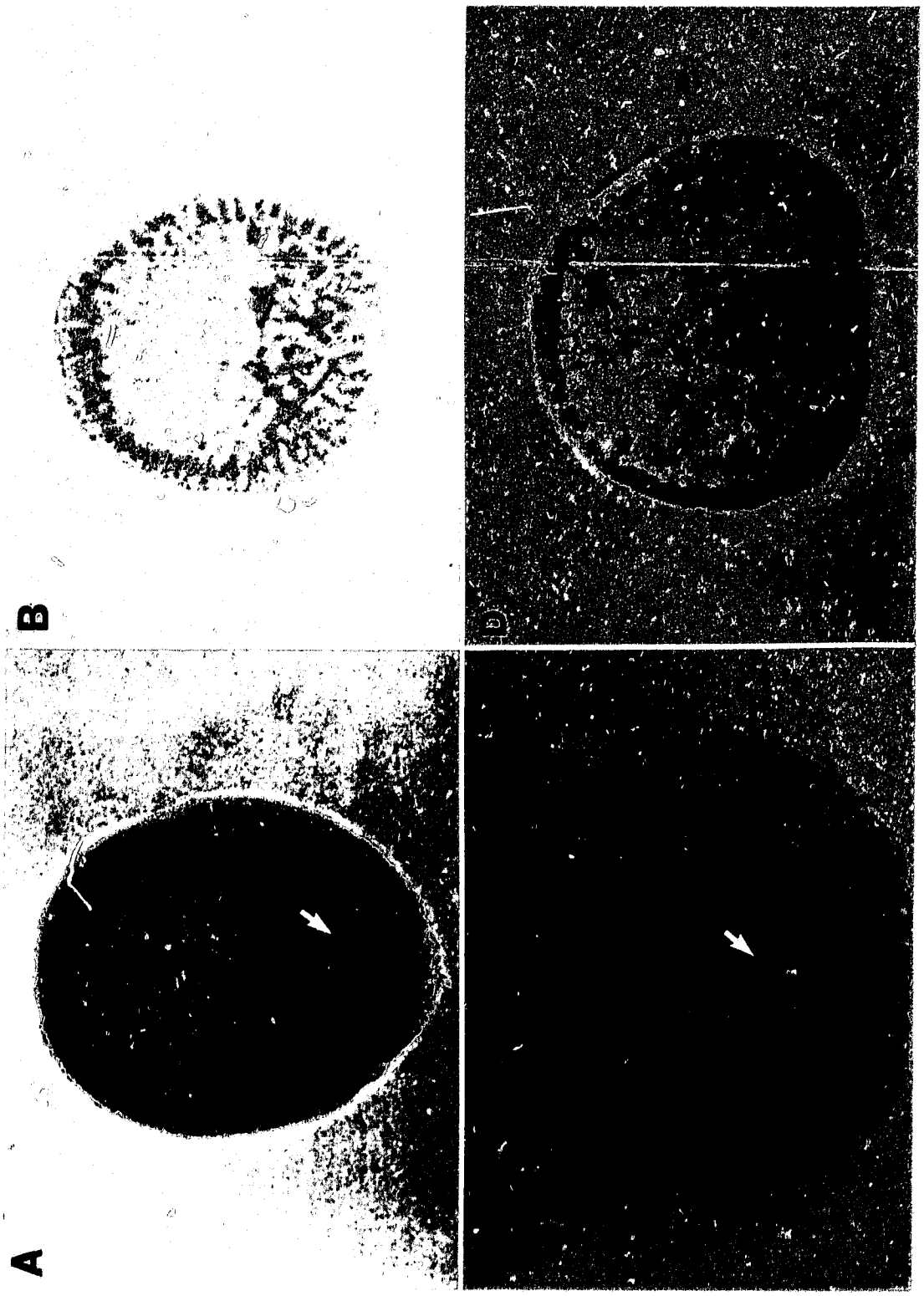

A
B
D

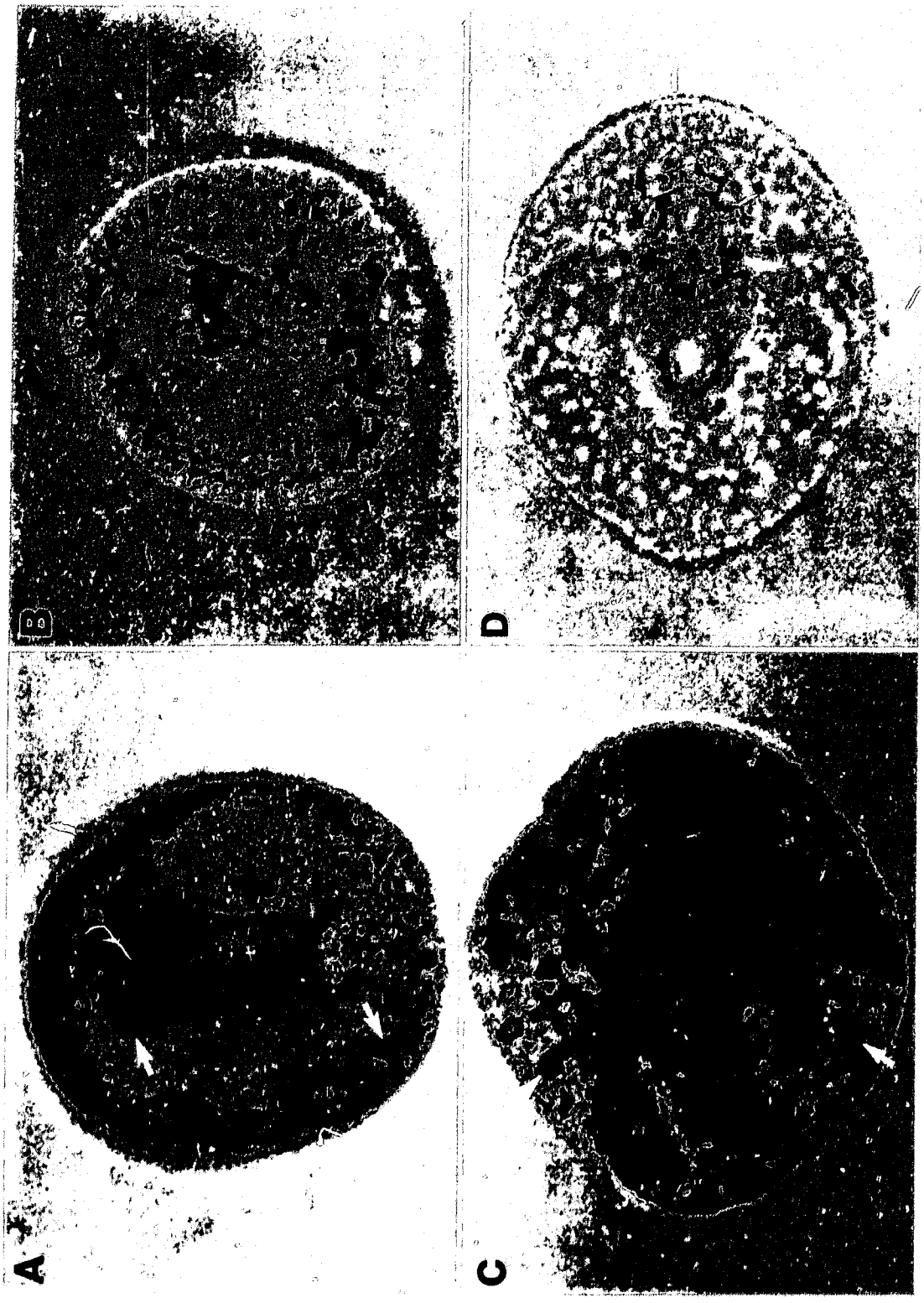
Figure 24: *In situ* analysis of late stage embryos with the βL subunit.

A. Late gastrula hybridized with antisense βL probe. The cells at the tip of the archenteron (s) and SMCs that have migrated in to the blastocoel express βL. A small numbre of cells in the vegetal region express low levels of βL (arrow).

B. Late gastrula hybridized with sense βL probe.

C. Prism hybridized with antisense βL probe (ventral view). A large number of blastocoelar cells express the βL subunit (arrows).

D. Prism hybridized with sense βL probe.


A
B
C
D

DISCUSSION

1.0 Sea urchin embryonic cells adhere and spread on Pronectin-F

Sea urchin embryos are easily dissociated in the absence of calcium and magnesium indicating that blastomere adherence is mediated via divalent cation dependant mechanisms. The integrins are one of the most prominent divalent cation dependant cell adhesion receptors. In an attempt to reveal integrin mediated cell adhesion events occurring in the early embryo, Pronectin-F, a substrate that contains a highly conserved integrin binding motif, was used in cell adhesion assays. Pronectin-F is a good candidate for this sort of experiment for a number of reasons. The GRGDS motif found in fibronectin has been cloned into the silk fibroin backbone with a spacing that mimics that which best supports RGD mediated cell adhesion (Protein Polymer Technologies literature). The density at which this motif is presented is much higher in Pronectin-F than it is in laminin or fibronectin containing substrates. Secondly, GRGDS is a redundant binding motif in the absence of peripheral sequences that are normally found in ECM components, and allows binding by a number of integrins with widely varying specificities (Loftus *et al.*, 1994). Thus this substrate in the absence of other ECM molecules was expected to mimic an RGD based ECM that could mediate integrin based cell adhesion in sea urchin embryos.

Cells isolated from various stages of post-cleavage embryos adhere to Pronectin-F. The cells isolated from midgastrula stage embryos display the greatest consistency of adhesion and were used in all the subsequent experiments. The reason why cells isolated from other stages of development adhere with such inconsistency is not clear. It may be

that in the earlier embryonic stages the cells that adhere are becoming competent to bind and that it is critical to exactly what stage the cells are isolated from the embryo. Isolation of cells too early may result in the absence of competence, or the loss of ability to respond to the influences that allow the development of competence to bind Pronectin-F, thereby reducing the observed number of adherent cells. At progressively later stages the embryos become more difficult to dissociate and the competent cells may be under represented if they remain as a coherent group and are removed by filtration before the cells are plated on Pronectin-F. It is also possible that the prolonged exposure of later stages to the dissociation medium required for dissociation has a negative effect upon cells, although microscopic examination of cells did not reveal any visible loss of cell integrity.

By rough estimates the adherent cell population is less than 5% of the cells in the embryo at the midgastrula stage of development and likely represents diverse populations as indicated by the morphologies of the adherent cells. This mixed population of cells does not attach to the control substrate consisting of the silk fibroin backbone alone suggesting the adhesive interaction is specific to the GRGDS sequence. The kinetics of attachment are slow with the maximum number of adherent cells being observed at four hours after plating. This delay in adhesion brings about the possibility that the cells are synthesizing a substrate that they subsequently adhere to. It is known that PMCs can synthesize a non-fibrillar collagen within 40 minutes of being isolated (Wessel *et al.*, 1991). However when these cells are detached from Pronectin-F with EDTA and plated back upon a Pronectin-F substrate they adhere and spread within 10 minutes suggesting

that adhesion is not mediated through the *de novo* synthesis of native ECM molecules. The slow time course that describes cell attachment may be a reflection of the incubation temperature which is 15° C or more below that used for vertebrate cells. The change in time course of attachment that is observed after preselection of cells on Pronectin-F suggests that the receptors used are not expressed, or are inactive on the dissociated cells at the time that they are initially plated. There are three possible explanations for this observation: the receptor(s) is not competent to bind the substrate, the receptor(s) is disrupted during the dissociation process, or that the receptor(s) is synthesized *de novo* after the cells encounter the substrate. In experiments such as these it is impossible to detect the change in the activation state of receptors as one has no knowledge of the receptors being utilized. The dissociation process is harsh and likely results in the denaturation of surface proteins. While it would have been desirable to use a dissociation medium that was less severe it was important to completely remove the native ECM to avoid the possibility that the cells are adhering to a substrate other than Pronectin-F. The possibility that the cells synthesize *de novo* a receptor that interacts with Pronectin-F is difficult to address. The actual number of adherent cells is low, limiting the possibilities for experimenting directly upon these cells. PCR was used in an attempt to differentially isolate β integrin subunit cDNAs from cells before and after adhering to Pronectin-F. The limiting amount of material presented a problem as the degenerate primers amplify histone RNAs in cDNA made from total RNA, and attempts to purify poly A+ RNA from attached cells was not successful. There are presently some newer methods available to isolate poly A+ RNA from as few as five cells and this offers the possibility that PCR

could be performed on these samples. However with no appreciable knowledge of integrin receptors in sea urchins it is unlikely that the mechanism mediating the adhesion of sea urchin cells to Pronectin-F can be easily deciphered.

1.1 Cells adhere to and spread on Pronectin-F using different mechanisms

Although cells adhere to Pronectin-F, it was important to provide evidence that this interaction was specific to the GRGDS sequence. The addition of a blocking peptide that mimics the GRGDS site does not result in appreciable decrease in the numbers of adherent cells but rather inhibits the spreading and migration of cells on Pronectin-F (Figure 4, 5). This suggests that attachment and spreading are separate events perhaps mediated by distinct mechanisms. It would appear that both events are mediated through the GRGDS motif as cells do not adhere to the silk fibroin backbone alone. How the GRGDS peptide distinguishes between the ability to spread and adhere is unknown. There are a number of lines of evidence that suggest that these two events are not directly connected. The addition of peptides to adherent cells can abolish the consequences of adhesion without preventing adhesion suggesting that the two events can be distinguished *in vitro* (Menko and Boettiger, 1987). Secondly, the growth of MMEs in a three dimensional matrix of laminin results in differentiated cells that exhibit no polarity but acquire the ability to make β-casein (Streuli *et al.*, 1991). *In vivo* these cells must form a polarized epithelium before they become competent to synthesize β-casein. Antibodies directed against β1 integrins abolish epithelium formation *in vivo* as well as β-casein production *in vivo* and *in vitro*. Thus it appears that it is possible to separate an adhesive

event from the morphological consequences of that event. The experiments utilizing MMEs do not distinguish between β1 integrins with different α subunits. It is possible that formation of an epithelium and induction of β-casein are mediated through different integrins, and a similar situation exists with the sea urchin cells that are adhering to Pronectin-F. Alternatively, the RGE control peptide used in these experiments is known to be inhibitory to a population of vertebrate integrins that bind RGD motifs suggesting that the recognition of this motif is not always strictly regulated when presented out of context (Smith, 1994; Loftus 1994). Therefore it is plausible that the GRGDS motif on Pronectin-F is recognized by a population of integrins that are insensitive to the GRGDS peptide. A number of integrins can bind to RGD containing peptides with varying affinities suggesting that this interaction is rather general in nature (Loftus *et al.*, 1994). This suggests that the RGD motif on Pronectin-F could support more than one activity through the interaction with multiple receptors. The complete inhibition of cell spreading by the GRGDS peptide suggests that this activity is mediated by an integrin that recognizes this motif with a high specificity, while attachment is perhaps more general in nature. Taken together these results suggest that more than one form of cell adhesion event is taking place in these assays. However the experiments do not distinguish between events being mediated by separate integrins or separate events being mediated through a single receptor.

1.2 Cells that adhere to Pronectin-F are not of mesodermal origin

Cells adherent on Pronectin-F display distinctive behaviors. The cells migrate extensively and fuse to form large syncytial structures (Figure 6). All indications are that the adherent cells are not of mesodermal origin as they are not recognized by mesoderm specific monoclonal antibodies (SP1, SP12) or by wheat germ aggulitinin (WGA), a marker for mesenchyme cells in the sea urchin embryo (DeSimone and Spiegel, 1986). Monoclonal antibodies raised against adherent cells do not recognize mesenchymal derivatives in whole embryos (Figures 7, 8). Thus it is likely that these cells are epithelial in origin and their behavior is aberrant being modified by the environment that they encounter. It was expected that due to the low number of cells that have the ability to adhere to Pronectin-F that these cells would represent a single population. However it is clear from the morphology of the cells that they represent a mixed population (Figure 5). There are no known examples of ectodermal cells becoming syncytial and it is likely that these cells are acquiring their phenotype as a result of the consequences of adhering to Pronectin-F. Rat fibroblasts acquire unique phenotypes when plated on immobilized RGD containing peptides. These cells display few if any stress fibers, and have structures that resemble point adhesions rather than focal contacts. The rat cells adhere slowly to peptide substrates as compared to fibronectin, but still spread and adhere strongly (Streeter and Rees, 1987). During cell adhesion assays the sea urchin cells are essentially plated on a substrate that consists of immobilized peptides with minimal conformation provided by the silk fibroin backbone. The sea urchin cells do not exhibit stress fibers but some thin actin fibrils can be found in cytoplasmic extensions (Figure 6). The cells appear

to adhere to Pronectin-F through a large number of point adhesions (Figure 6). These are found in cells that are spread and in those that remain rounded and may reflect the site of adhesion to Pronectin-F. This evidence would suggest that the substrate is regulating the phenotype of the sea urchin cells. The kinetics of attachment would seem to indicate that this phenotype is acquired upon exposure to the substrate, either through gene expression or activation of a receptor that can utilize the substrate. The evidence presented here does not rule out other mechanisms, however there is precedence for adherence to specific ECM components resulting in the acquisition of fate (Streuli *et al.*, 1991).

Some of the cells resemble those observed by others when SMCs are plated on glass (Jeff Hardin, personal communication). It is unlikely that these cells are SMCs as the procedure used to coat coverslips should ensure that the no glass is available for cell attachment. The coverslips are coated with silicone before being incubated in the presence of Pronectin-F. In control experiments no cells adhere to silicone treated coverslips (data not shown). The other possibility is that the blocking agent (BSA) allows for cell adherence. BSA coated coverslips do allow for some cell attachment but these cells are static and appear as bipolar cells (data not shown). Control experiments using Beta-Silk confirm that the attachment observed is specific to the substrate as few if any cells adhere to Beta-Silk (Figure 3).

1.3 Cells adherent upon Pronectin-F are of epithelial origin

Monoclonal antibodies were raised against membrane preparations isolated from attached cells in an attempt to localize these epitopes in the whole embryo. A number of

antibodies were selected for further analysis based upon their ability to recognize epitopes both in whole embryos and as immunoprecipitates (Figure 7). None of the 3500 hybridoma fusions secreted antibody that recognized mesenchymal epitopes. The antibody 8F2 recognizes an epitope that is localized to the apical periphery of all epithelial cells in the post-cleavage sea urchin embryo (Figure 8). Immunoprecipitations of cell surface labelled material indicate that the epitope is localized to the cell surface (Figure 7). The method used to label cells in this instance does not allow for the immunoprecipitation of dimeric molecules as the dissociation process denatures most surface molecules (Fink and McClay, 1984). The procedure is further complicated by the reactivity of the labelling compound with the normal constituents of sea water. Kinetic labelling of cells may provide a better mechanism for obtaining labelled molecules. When the antibodies are used on western blots the results are inconclusive as some preparations give a high molecular weight band while others show no immunoreactivity (data not shown). While further characterization is needed before any conclusions can be drawn about the identity of this epitope, the antibody does display some interesting developmental regulation of expression.

The distribution of the 8F2 epitope is modified as development progresses and the domain of expression is restricted to the oral surface and presumptive digestive tract after the archenteron fuses with the ectoderm (Figure 8). The progressive restriction in the domain of expression of the 8F2 molecule is hard to correlate to tissue specific expression. It is expressed in endodermal as well as ectodermal derivatives. In the ectoderm it is found in the ciliary band that develops from both the oral and aboral

ectoderm (Cameron, 1993). This indicates that this molecule is regulated in a fashion that crosses germ layer boundries as well as those defined by the five initial domains established during cleavage. The localization of this epitope is epithelial and therefore localized to cells that remain adherent during cellular rearrangements suggesting that the 8F2 antigen is likely to mediate cell attachment. Addition of 8F2 antibodies to adhesion assays has no observable effect on cell behavior, and when embryos are grown in the presence of the antibody there is no effect upon development. These experiments support the idea that the 8F2 antigen does not mediate cell movement. At this time there is no evidence to the identity of the 8F2 antigen but the localization would suggest that it is involved in cell/cell interactions within epithelial sheets. The *Drosophila* βv integrin localizes to epithelial sheets in the developing midgut and it is thought that it acts to maintain epithelial integrity during morphogenetic movements (Yee and Hynes, 1993). If the 8F2 antigen played a similar role it is possible that this molecule could mediate cell/cell interactions that lead to fusion *in vitro* when cells are adherent upon a foreign substrate.

The antibodies have not been used to attempt to identify cells in adhesion assays. These experiments would help in clarifying if the separate monoclonals identify unique cell populations in the adherent cells. It is assumed that the cells that have distinctive morphologies represent diverse populations in the intact embryo. If this turns out to be the case then these antibodies may provide markers for cell lineages or embryonic cell populations.

2.0. The sea urchin βC integrin subunit

While the βC subunit is an incomplete clone (Figure 11) and as a consequence there is not a great amount of comparison that can be done to existing integrins, it is useful to examine the relationships between this subunit and the vertebrate subclasses that are recognized. Ransom *et al.* (1993) have shown that when compared over the ligand binding domain the percentage identity of integrin β subunits is similar to that of the whole subunit. With this in mind the βC clone, which encompasses most of the ligand binding domain, appears to be unique when compared to the known vertebrate subclasses (Table 2). The βC subunit bears the closest resemblance to the other sea urchin β subunits (27-30%), although the similarity would not appear to be significantly greater than that for the vertebrate β subunits (26%). The βC subunit outclasses with the β8 and β4 subunit when it is included in a dendrogram as in Figure 25. In this figure the βC subunit is not included as the sequence is incomplete. The percentage identities (Table 2) support the conclusion that the βC subunit is unique and likely is not the homologue of any known vertebrate β subunit.

The uniqueness of the βC subunit is apparent in the primary sequence (Figure 11). The conserved motif **DXSXSXXDXXN/S** found in the ligand binding domain of all integrins is modified in βC to **DLSDSMSDDLV** (Figures 11, 26). This sequence is also modified in another sea urchin β integrin subunit, βG, to include a glutamine in the last site of the consensus (Figures 14, 26). In βG this is immediately followed by an asparagine residue suggesting that there may be some flexibility to the length of the

consensus. In the βC subunit this cannot be the case as the final position of the consensus is a valine and there is no serine or asparagine within the neighboring residues to account for such flexibility (Figure 11). In the middle of the ligand binding domain at the consensus sequence I/LGFGS, the phenyalanine residue is replaced by a tyrosine. While this is a conservative substitution, βC is the only β integrin in which this amino acid is not a phenyalanine. The ligand binding domain of the sea urchin β subunits appear to have runs of amino acids that are similar (Figure 26) and the βC subunit bears approximately equal resemblance to both of the other sea urchin β subunits.

The location of the cysteines that are found in the βC fragment are of interest as the cysteine residue in position 11 appears to be unique. This cysteine does not align with a cysteine residue found in any other known β integrin subunit. In the other sea urchin β subunits there are cysteine residues that are in unique positions (Figure 26, cysteine 12 in βG and βL). In the βG and βL subunits a cysteine residue at another location is lost to compensate for the novel cysteine (Figure 26). All the cysteine residues in βC, with exception of cysteine 11, are in conserved positions (Figure 26). This suggests that if there is a cysteine residue lost that it must be in the last 5 cysteines of βC.

On northern blots the βC transcript is detected as a 7.5 Kb RNA that is present as a maternal transcript in the egg (Figure 12). During cleavage there is a small increase in the prevalence of this transcript that decreases after cleavage to low levels. The decrease in expression of this molecule after cleavage would suggest that this is the time in development during which this molecule is active. There is no information available on

the distribution of this transcript in the embryo, however at this stage the possible roles played by this molecule are limited.

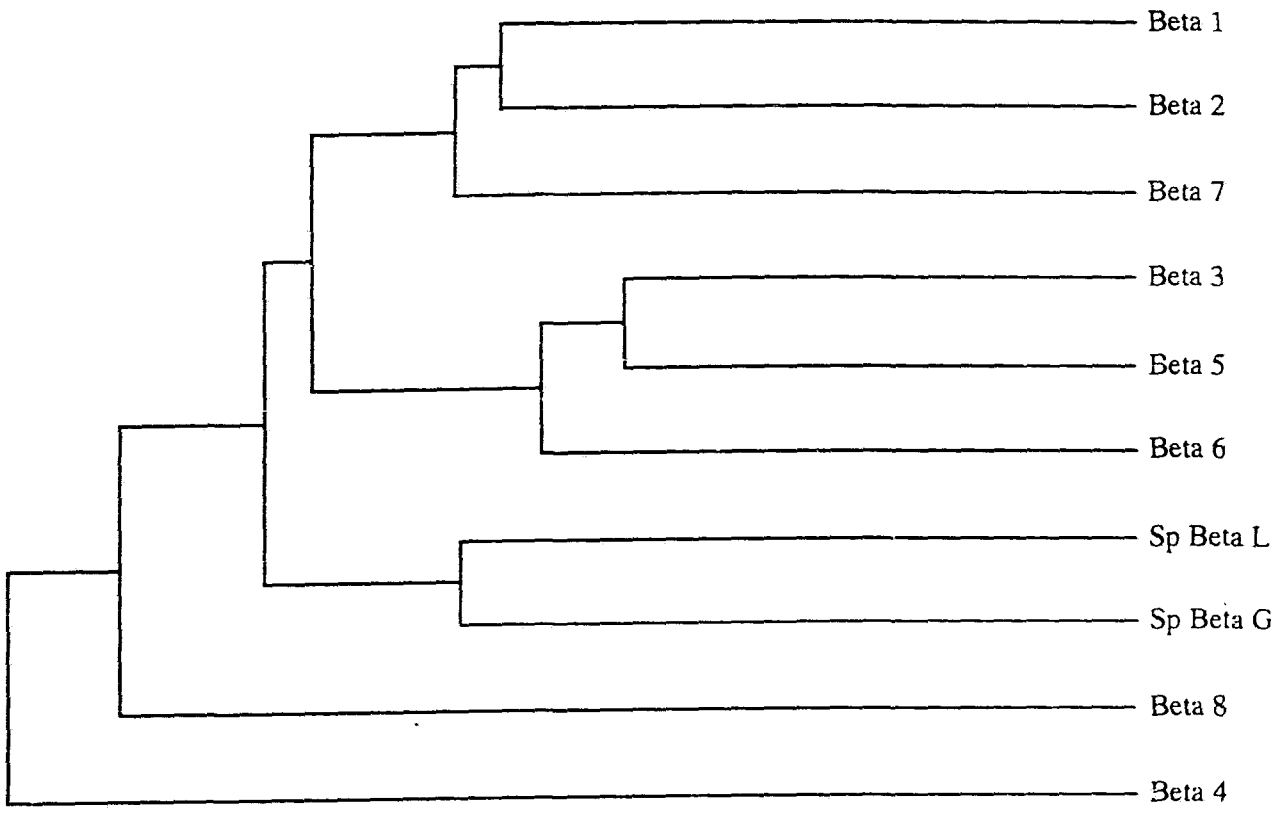
3.0 The sea urchin βG integrin subunit

The βG subunit was identified as a 2577 bp open reading frame. The primary structure of the βG subunit is highly conserved and is approximately equally related to all other known β subunits (Table 2). It is interesting that the βG subunit does not fit into the proposed classification scheme for vertebrate inetgrins (Figure 25). βG bears the closest resemblance to the β1 (40%), β3 (38%), and β6 (36%) integrins (Table 2). However, the vertebrate β1, β3, β6 integrins do not group together based upon their sequence similarity (Figure 25). Considering these relationships it is unlikely that the βG subunit is the homologue of any vertebrate integrin. There are however a number of sequences in the βG subunit that suggest that it is most closely related to the β1 subunits.

The putative ligand binding domain of βG extends from amino acid 133-371, and as expected displays a high degree of similarity to other known β integrins. The aspartic acid residue at position 141 is absolutely conserved in all known β integrins and is thought to act in ligand binding (Loftus *et al.*, 1990). The sequence **DLSKSMEDDLS** (amino acids 141-151) in the extracellular portion of βG (Figures 14, 26) represents a consensus sequence **DXSXSXXDXXN/S** of oxygenated residues that are thought to act in coordination of divalent cations in β subunits (Loftus *et al.*, 1990). Unlike the other sea urchin β subunits this sequence is completely conserved in βG. It is this sequence that

Figure 25: Dendrogram of relatedness of β integrin subunits.

Only the extracellular portions of β subunits was used for the comparison. Comparisons used to generate the figure are the same as those in Table 2. Figure was generated using the Gene Works program (Intelligenetics).

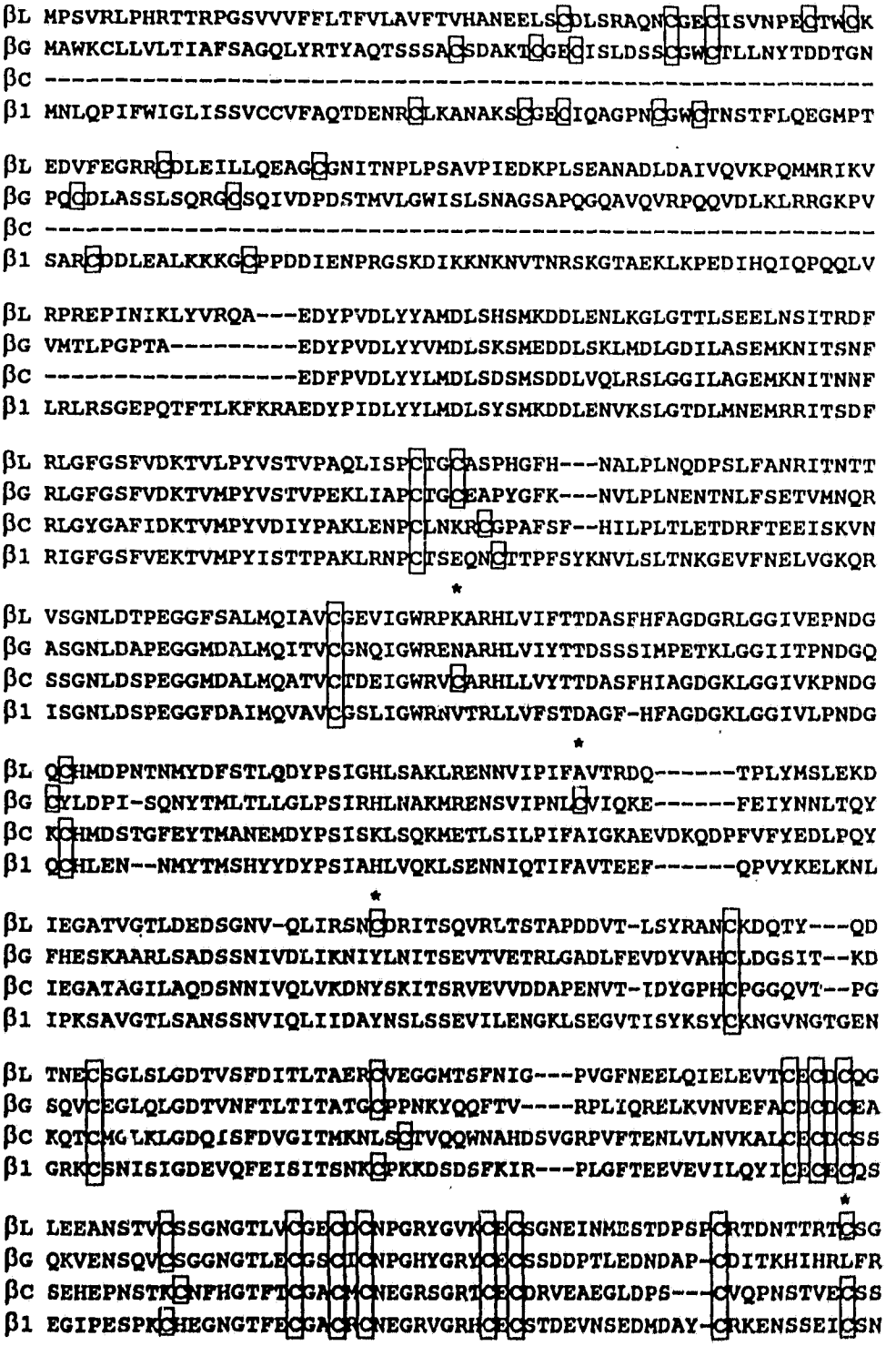

Beta 1
Beta 2
Beta 7
Beta 3
Beta 5
Beta 6
Sp Beta L
Sp Beta G
Beta 8
Beta 4

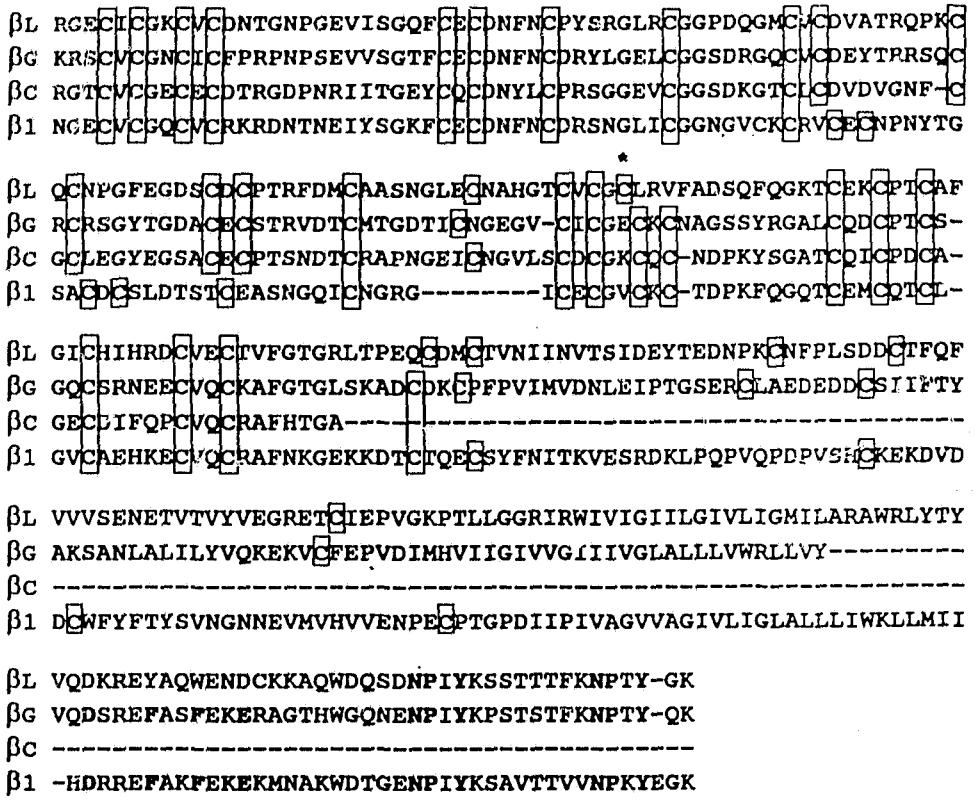
has been implicated in the exchange of a divalent cation for ligands that contain a conserved aspartic acid residue. With this in mind it is possible that the ligand for the βG subunit contains such a motif (see later discussion).

The extracellular domain contains the conserved 56 cysteine residues that are found in most β integrins (Figures 14, 26). The location of these cysteines is highly conserved in β integrins and is thought to act in establishing secondary structure through disulphide bonds (Calvete *et al.*, 1991). The cysteines found in the ligand binding domain of the βG subunit are highly conserved and presumably this reflects the formation of secondary structure essential to establishing the dimeric ligand binding site. Cysteine 12 is out of register with cysteine 12 found in vertebrate integrins (Figure 26). Presumably this cysteine replaces the missing cysteine that would normally be located at position 45 (Figure 26). This is supported by the observation that cysteine 13 of βG aligns with cysteine 12 of the vertebrate integrins (Figure 26). Unlike the cytoplasmic cysteines, those found outside the cell are susceptible to interactions with numerous substances and therefore are only found as pairs (Alberts *et al.*, 1994). In the β4, β7, β8, and βv subunits there are fewer than 56 cysteines found in the extracellular domain, however there are always a even number of cysteines. The missing cysteines are revealing to the location of the disulphide bonds that create secondary structure as they are presumably lost as pairs. Interestingly, neither the cysteine at position 12 nor that at 45 are among those that are lost in other subunits (Erle *et al.*, 1991; Moyle *et al.*, 1991; Yee and Hynes, 1993). Calvete *et al.*, (1991) propose that cysteine 12 and cysteine 45 of the vertebrate β integrins interact with immediate neighbors. The shift of the location of the cysteine 12 of

Figure 26: Alignment of the sea urchin β subunits with the human β1 subunit

The human β1 subunit was aligned with the sea urchin β subunits to show the relative positioning of the cysteine residues in the extracelllular domain. The positioning of the cysteines in the vertebrate β subunits is highly conserved and the β1 subunit was chosen as a representative example as the βG and βL subunits bear the closest identity to this vertebrate β integrin subunit. The cysteine residues are boxed for clarity. Asterisks above the sequence mark sites where the sea urchin β subunits contains insertions or deletions of cysteine residues. Conserved residues found in the cytoplasmic domain of vertebrate β1 integrins are in bold. Alignment was done using the Gene Works program (Intelligenetics).


βL MPSVRLPHRTTRPGSVVVFFLTFVLAVFTVHANEELSCDLSRAQNCGECISVNPECTWCK
βG MAWKCLLVLTIAFSAGQLYRTYAQTSSSACSDAKTCGECISLDSSCGWCTLLNYTDDTGN
βC ----------
β1 MNLQPIFWIGLISSVCCVFAQTDENRCLKANAKSCGECIQAGPNCGWCTNSTFLQEGMPT
βL EDVFEGRRCDLEILLQEAGCGNITNPLPSAVPIEDKPLSEANADLDAIVQVKPQMMRIKV
βG PQCDLASSLSQRGCSQIVDPDSTMVLGWISLSNAGSAPQGQAVQVRPQQVDLKLRRGKPV
βC ----------
β1 SARCDDLEALKKKGCPPDDIENPRGSKDIKKNKNVTNRSKGTAEKLKPEDIHQIQPQQLV
βL RPREPINIKLYVRQA---EDYPVDLYYAMDLSHSMKDDLENLKGLGTTLSEELNSITRDF
βG VMTLPGPTA---------EDYPVDLYYVMDLSKSMEDDLSKLMDLGDILASEMKNITSNF
βC ----------EDFPVDLYYLMDLSDSMSDDLVQLRSLGGILAGEMKNITNNF
β1 LRLRSGEPQTFTLKFKRAEDYPIDLYYLMDLSYSMKDDLENVKSLGTDLMNEMRRITSDF
βL RLGFGSFVDKTVLPYVSTVPAQLISPCTGCASPHGFH---NALPLNQDPSLFANRITNTT
βG RLGFGSFVDKTVMPYVSTVPEKLIAPCTGCEAPYGFK---NVLPLNENTNLFSETVMNQR
βC RLGYGAFIDKTVMPYVDIYPAKLENPCLNKRCGPAFSF--HILPLTLETDRFTEEISKVN
β1 RIGFGSFVEKTVMPYISTTPAKLRNPCTSEQNCTTPFSYKNVLSLTNKGEVFNELVGKQR
βL VSGNLDTPEGGFSALMQIAVCGEVIGWRPKARHLVIFTTDASFHFAGDGRLGGIVEPNDG
βG ASGNLDAPEGGMDALMQITVCGNQIGWRENARHLVIYTTDSSSIMPETKLGGIITPNDGQ
βC SSGNLDSPEGGMDALMQATVCTDEIGWRVCARHLLVYTTDASFHIAGDGKLGGIVKPNDG
β1 ISGNLDSPEGGFDAIMQVAVCGSLIGWRNVTRLLVFSTDAGF-HFAGDGKLGGIVLPNDG
βL QGHMDPNTNMYDFSTLQDYPSIGHLSAKLRENNVIPIFAVTRDQ------TPLYMSLEKD
βG GYLDPI-SQNYTMLTLLGLPSIRHLNAKMRENSVIPNLGVIQKE------FEIYNNLTQY
βC KGHMDSTGFEYTMANEMDYPSISKLSQKMETLSILPIFAIGKAEVDKQDPFVFYEDLPQY
β1 QGHLEN--NMYTMSHYYDYPSIAHLVQKLSENNIQTIFAVTEEF------QPVYKELKNL
βL IEGATVGTLDEDSGNV-QLIRSNCDRITSQVRLTSTAPDDVT-LSYRANCKDQTY---QD
βG FHESKAARLSADSSNIVDLIKNIYLNITSEVTVETRLGADLFEVDYVAHCLDGSIT--KD
βC IEGATAGILAQDSNNIVQLVKDNYSKITSRVEVVDDAPENVT-IDYGPHCPGGQVT--PG
β1 IPKSAVGTLSANSSNVIQLIIDAYNSLSSEVILENGKLSEGVTISYKSYCKNGVNGTGEN
βL TNECSGLSLGDTVSFDITLTAERCVEGGMTSFNIG---PVGFNEELQIELEVTCECDCQG
βG SQVCEGLQLGDTVNFTLTITATGCPPNKYQQFTV----RPLIQRELKVNVEFACDCDCEA
βC KQTCMGLKLGDQISFDVGITMKNLSCTVQQWNAHDSVGRPVFTENLVLNVKALCECDCSS
β1 GRKCSNISIGDEVQFEISITSNKCPKKDSDSFKIR---PLGFTEEVEVILQYICECECQS
βL LEEANSTVCSSGNGTLVCGECDCNPGRYGVKCECSGNEINMESTDPSPCRTDNTTRTCSG
βG QKVENSQVCSGGNGTLECGSCICNPGHYGRYCECSSDDPTLEDNDAP-CDITKHIHRLFR
βC SEHEPNSTKCNFHGTFTCGACMCNEGRSGRTCECDRVEAEGLDPS---CVQPNSTVECSS
β1 EGIPESPKCHEGNGTFECGACRCNEGRVGRHCECSTDEVNSEDMDAY-CRKENSSEICSN


βL RGECICGKCVCDNTGNPGEVISGQFCECDNFNCPYSRGLRCGGPDQGMCVCDVATRQPKC
βG KRSCVCGNCICFPRPNPSEVVSGTFCECDNFNCDRYLGELCGGSDRGQCVCDEYTRRSQC
βC RGTCVCGECECDTRGDPNRIITGEYCQCDNYLCPRSGGEVCGGSDKGTCLCDVDVGNF-C
β1 NGECVCGQCVCRKRDNTNEIYSGKFCECDNFNCDRSNGLICGGNGVCKCRVCECNPNYTG
βL QCNPGFEGDSCDCPTRFDMCAASNGLECNAHGTCVCGCLRVFADSQFQGKTCEKCPTCAF
βG RCRSGYTGDACECSTRVDTCMTGDTICNGEGV-CICGECKCNAGSSYRGALCQDCPTCS-
βC GCLEGYEGSACECPTSNDTCRAPNGEICNGVLSCDCGKCQC-NDPKYSGATCQICPDCA-
β1 SACDCSLDTSTCEASNGQICNGRG--------ICECGVCKC-TDPKFQGQTCEMCQTCL-
βL GICHIHRDCVECTVFGTGRLTPEQCDMCTVNIINVTSIDEYTEDNPKCNFPLSDDCTFQF
βG GQCSRNEECVQCKAFGTGLSKADCDKCPFPVIMVDNLEIPTGSERCLAEDEDDCSIIFTY
βC GECDIFQPCVQCRAFHTGA----------
β1 GVCAEHKECVQCRAFNKGEKKDTCTQECSYFNITKVESRDKLPQPVQPDPVSRCKEKDVD
βL VVVSENETVTVYVEGRETCIEPVGKPTLLGGRIRWIVIGIILGIVLIGMILARAWRLYTY
βG AKSANLALILYVQKEKVCFEPVDIMHVIIGIVVGIIIVGLALLLVWRLLVY---------
βC ----------
β1 DCWFYFTYSVNGNNEVMVHVVENPECPTGPDIIPIVAGVVAGIVLIGLALLLIWKLLMII
βL VQDKREYAQWENDCKKAQWDQSDNPIYKSSTTTFKNPTY-GK
βG VQDSREFASFEKERAGTHWGQNENPIYKPSTSTFKNPTY-QK
βC ----------
β1 -HDRREFAKFEKEKMNAKWDTGENPIYKSAVTTVVNPKYEGK

the βG subunit suggests that this assumption is incorrect as all other cysteines retain conserved positions.

The extracellular domain contains eight potential N-glycosylation sites (Figure 14) and if one assumes an average addition of 2.5 kDa for each processed N-glycosylation site (Tamura *et al.*, 1990) this would account for the disparity between the 90 kDa predicted molecular mass and the observed predicted molecular mass of 120 kDa on reducing SDS-PAGE gels (figure 16).

The cytoplasmic domain is 39 amino acids long and contains a conserved tyrosine at position 781 that is phosphorylated in β1 integrin (Figure 14, DeSimone and Hynes, 1988). The cytoplasmic domain also contains a number of residues that are known to be essential in the attachment of fibroblasts to the ECM through the binding of β1 integrins (Figure 26, DSREFASFEKERAGTHWGQNENPIYKPSTSTFKNPTYQK) (Reszka *et al.*, 1992). The sequence **NPXY** , which is present twice in the cytoplasmic domain of βG (Figures 14, 26) is thought to form a dimerized region in vertebrate B1 integrins that interacts with talin in focal contacts (Lewis and Schwartz, 1995). The tyrosine residue at position 781 (Figure 14) has been implicated in phosphorylation mediated receptor inactivation and internalization (Tamkun *et al.*,86; Chen *et al.*, 1990). The presence of these residues suggests that βG may represent a homologue of the vertebrate β1 integrins. The cytoplasmic region also contains a serine residue at position 774 (Figure 14) that corresponds to the serine that is required in human β3 integrins for activation of the receptor by intracellular signals (Chen *et al.*,1992). This serine residue is also present

in the β7 (Erle *et al.*, 1991) and β8 (Moyle *et al.*, 1991) integrins but it is not known if it is essential for the function of receptors that contain these subunits.

The βG subunit is encoded by a 7.5 kb transcript that contain extensive untranslated flanking regions or possibly an extremely long poly A+ tail as the coding sequence comprises less than one third of the transcript length. Due to the extreme length of the flanking regions no attempt was made to characterize these regions. There is a low level of transcript detected in unfertilized eggs (Figure 15). This may represent a basal level of expression as it appears to be similar in all the subunits (Figures 12, 15, 21). The prevalence of the transcript increases slowly until the midgastrula stage of development and then decreases (Figure 15). The high levels of expression during gastrulation may be the result of overloading of the gastrula stage lane on the Northern blot. While the increase in amount of RNA in this lane is not great, it may be enough to give the impression that there is an increase in expression at this time. Evidence from *in situ* localization would suggest that the levels of expression of the βG subunit remain essentially constant during the later stages of development (Figure 18), although estimates from this type of data are not precise. The prevalence of the transcript appears to be low at all times in development as the signal is never intense on Northern blots, even after prolonged exposures. The same blots were used to probe for the presence of the fibropellin transcript (Bisgrove and Raff, 1993) and strong signals were obtained suggesting that the integrin subunittranscripts do not accumulate. The use of more sensitive quantitative techniques such as ribonuclease protection assays would allow for better comparative estimation of transcript prevalence. The peak in expression of the βG

transcript at gastrulation suggests that this molecule plays a role at this time. This assumption is based upon the relative levels of the transcript, however β integrins are known to be regulated primarily at the post-translational level making an assessment of function based upon the presence of the transcript difficult (deStrooper *et al.*, 1991).

A polyclonal antiserum was raised against a portion of βG expressed in bacteria. The expressed portion of the molecule encompasses the dimeric ligand binding site and was selected to include as few glycosylation sites and cysteine bonded areas as possible. On western blots of extracts prepared from membrane preparations run in the presence of non-reducing sample buffer the 61CR serum recognizes a series of bands that migrate at an apparent molecular weight of 116-120 kDa (Figure 16). It is unclear if the number of bands observed represents a number of distinct integrins, or if this banding pattern is a result of different structures representing the same protein. There are a number of lines of evidence that suggests 61CR recognizes a single protein. It has been observed that the cysteine bonding pattern in isolated integrins is labile (Loftus *et al.*, 1994) which would allow for a number of forms being observed in non-reducing western blots. In these experiments the extracts used for the western blots are made up in TBS as the salt concentrations found in sea water cannot be run on SDS-PAGE gels. There is evidence that lower salt concentrations cause non-reversible changes to the secondary structure of integrins resulting in various amounts of decrease in secondary structure and hence the appearance of a variety of forms (Steiner *et al.*, 1992) Another possibility is that the secondary structure of the non-reduced integrin does not allow easy access for SDS and this results in an apparent heterogeneity when separated by SDS-PAGE. An alternative

explanation is that the forms observed represent molecules with different amounts or types of glycosylation. Changes in glycosylation are typically characterized by shifts in an apparent molecular weight of 10-15 kDa which correlates well with the results obtained here. When the extracts are run in the presence of reducing sample buffer a total of three bands are observed at all stages of development with the exception of blastula. The relative distribution of these bands is altered as development proceedes. It is known that in both T lymphocytes (Wadsworth *et al.*, 1993) and keratinocytes (Kim *et al.*, 1991) developmentally regulated differential glycosylation results in the appearance of distinct forms of the β1 integrin subunit. During *Xenopus* development a variety of forms of the β1 integrin are observed and this has been attributed to different levels of glycosylation (Gawatanka *et al.*, 1992). The presentation of β1 integrin subunits on the surface of cells requires the precursor molecule to be glycosylated and the mature receptor is glycosylated an additional time just before export to the surface (Akiyama *et al.*, 1989). This glycosylation event often results in two distinct forms of the receptor being observed on immunoblots. Increasing amounts of reducing agent in sample buffer causes the high molecular weight bands to disappear and the appearance of a band with an apprent molecular weight of 60 kDa. The 60 kDa band is not removed by preabsorbtion of the antisera with *E. coli* lysates, but is not apparent after preabsorbtion with the expressed fragment suggesting the recognition is specific (data not shown). The 60 kDa band may be due to proteolysis of the βG subunit subsequent to reduction of disulphide bonds in reducing sample buffer. While the membrane extracts are prepared in a protease inhibitor cocktail this may not be sufficient to protect integrins from degradation as it has been

observed that reduced integrins are susceptible to proteolysis even in the presence of a proteinase inhibitor cocktail (Loftus *et al.*, 1994; Hynes, 1992). In an attempt to minimize proteinase activity the reduced samples were run in a minimal amount of β-mercaptoethanol. It is worthy to note that reduction of the disulphide bonds in β integrins is difficult and that reducing agent concentrations below optimal levels cause rearrangement of bonds rather than complete reduction (Phillips and Agin, 1977) and the use of lower concentrations of β–mercaptoethanol may possibly contribute to the observation of multiple bands on the western blots.

The translational control over the expression of the βG integrin mimics the transcriptional pattern (Figures 15, 16). This is different from other β integrins in which control of expression is primarily post-transcriptional (DeStrooper *et al.*, 1991). There are small amounts of βG found as maternal protein on eggs, and this low level of expression is maintained throughout cleavage and into the blastula stage of development (Figure 16). During gastrulation, there is a dramatic increase in the expression of βG that is carried through into the pluteus stage of development where the down regulation of expression is reflected in a decrease in intensity of staining on western blots (Figure 16). The decrease in transcript prevalence at this time would be in accordance with the result observed on western blots. Since membrane preparations were used to make lysates, the signals observed on western blots probably represent primarily integrins that are expressed on the surface of the cells and not cytoplasmic stores of the protein. While immunoprecipitations of cell surface labelled material would clarify this issue, labelling of the cell surface in sea urchin embryos is complicated by the presence of the hyaline layer

and the apical lamina which inhibit labelling of cell surface molecules. When embryos are dissociated and surface labelled it is unlikely that dimeric integrins would be recovered due to the harsh procedure required for dissociation. One can use ^{35}S-methionine to incorporate label into newly synthesized integrin subunits, but this approach does not distinguish between cell surface molecules and those stored in the cytoplasm. Thus these experiments are difficult and may require the development of novel approaches.

3.1 Expression of βG is correlated with formation of the archenteron

In situ localizations were used to gain an estimation of the spatial regulation of the βG subunit. The transcripts are localized to the developing archenteron from invagination through until the early pluteus stage (Figures 17, 18). The staining intensity during development remains essentially constant although at all stages it is weak. The epithelium that forms the archenteron is derived from the vegetal plate and does not lose its integrity during formation of the archenteron. While there is no evidence what role the βG plays in archenteron formation perhaps it assists in maintaining epithelial integrity. The SMCs and pigment cells detach from the archenteron during the time it extends across the blastocoel. The βG subunit is expressed in the archenteron but not the SMCs (Figure 18), suggesting that it is not active in cell migration in this population of cells. The cells that comprise the archenteron do undergo movements that mimic those of migrating cells. The cells produce extensive filopodial extensions that appear to be responsible for the convergence-extension movements that drive the secondary phase of archenteron extension (Hardin, 1989). The expression of endo16, an ECM molecule that contains a

conserved integrin binding motif, RGD, is closely correlated with the formation of the archenteron (Soltysikespanola *et al.*, 1994), but is not associated with other derivatives of the vegetal plate. One would expect that given the spatially restricted expression of an ECM component that contains a conserved integrin binding motif, that one would find the expression of an integrin receptor in these areas. Perhaps βG represents the β subunit of such a putative receptor.

3.2 βG is associated with pigment cell migration

The only other location in which the βG subunit is found is in the pigment cells (Figure 18). These cells detach from the archenteron during the primary phase of gastrulation and migrate throughout the blastocoel until late gastrula when they begin to invade the ectoderm (Gibson and Burke, 1985). These cells stain intensively for the βG subunit during the later phases of migration (Figure 18) and remain positive for the βG transcript immediately after insertion into the ectoderm. Integrins are known to act in cell migration and there is ample evidence that these molecules mediate the extensive migrations observed in chick neural crest cells (Bonner-Fraser, 1994). While neural crest cells appear to migrate along defined pathways that are previously established (Fosney *et al.*, 1994), the pigment cells appear to wander in the blastocoel and eventually take up widely spaced locations (Gibson and Burke, 1985). The invasion of the ectoderm by the pigment cells could be viewed as being analogous to the extravasation of neutrophils across the circulatory system endothelium, an activity that is dependant upon β2 integrins. In this situation the leukocytes express β2 integrins on their surface yet the receptors are

inactive until the leukocytes comes into close contact with the endothelium (reviewed in Hynes, 1992). Thus the site of extravasation is established locally and not by predetermined pathways. Perhaps the pigment cells migrate until they become associated with a localized molecule that allows for activation of a receptor that allows for static adhesion. As discussed below the βL subunit is also associated with pigment cells late in development, and there is a possibility that the two subunits act cooperatively to allow for localization, and invasion of the ectoderm.

4.0. The βL Subunit

The extracellular domain of the βL subunit resembles that of the βG subunit (43% similarity) more than it does those of the known vertebrate β integrins. Like the βG subunit, the βL subunit does not fit the classification scheme presented in the dendrogram of Figure 25. While βL bears the closest resemblance to human β1 subunit (40%), it bears approximately equal similarity to the β3 (38%) subunit (Table 2). This would seem to confirm that the sea urchin integrins are distinct from those of the vertebrates. Interestingly the sea urchin β integrins are no more closely related to the other invertebrate integrins than they are to the vertebrate integrins.

A significant departure from the known integrins is the difference observed in the oxygenated residue consensus sequence **DXSXSXXDXXN/S** found in the ligand binding domain of all other known β integrins (Loftus *et al.*, 1990). In βL this sequence is represented by amino acids 147-157, **DLSHSMKDDLE** with the final amino acid not matching the consensus sequence (Figure 20). Amino acid 158 is an asparagine so the

deviation from the consensus sequence may not be functionally significant. The completely conserved aspartic acid residue that is thought to act in ligand binding is found at the beginning of the consensus (position 147, Figure 20).

The 56 conserved cysteine residues are found in the extracellular domain of βL (Figures 20, 26). The spacing of the cysteine residues is similar to that observed in the βG subunit. The cysteine at position 12 does not align with the cysteine 12 of the βG subunit, however it is found in a similar region of the molecule (Figure 26). As described for the βG subunit the novel location of cysteine 12 results in the loss of a cysteine residue elsewhere in the molecule. In βL the lost cysteine is equivalent to the vertebrate cysteine at position 26 (Figures 20, 26). Calvete *et al.* (1991) propose that this cysteine forms a disulphide bond with cysteine 28 in the vertebrate integrins. As the cysteine at position 28 is conserved in βL (Figure 26) the disulphide bonding pattern must deviate from that hypothesized by Calvete *et al.* (1991).

There are seven N-glycosylation sites in the βL molecule, one less than found in βG (Figure 20). The expected molecular weight of the βL subunit is 88 kDa and the observed molecular weight of 120 kDa (Figure 22) suggests that this is due to the glycosylation of the molecule. Glycosylation at these seven sites would add approximately 18 kDa to the protein (Tamura *et al.*, 1990) and this is about two thirds of the difference between the estimated and observed molecular weights.

The 39 residue long βL subunit cytoplasmic domain is structurally similar to that of βG (figure 26). It contains many of the residues that are thought to act in organizing the actin cytoskeleton including the duplicated **NPXY** sequence at amino acids 785-788

and 796-799 thought to interact with talin (Lewis and Schwartz, 1995). The consensus sequence proximal to the transmembrane domain that is found in β1 integrins and thought to mediate the adhesion to the ECM (Reska *et al.*, 1992) is not found in the βL subunit (figure 26). Due to the lack of these highly conserved sequences it is unlikely that the βL subunit is a homologue of the β1 integrin subunit. The βL subunit does have the conserved tyrosine residue phosphorylated in β1 integrins (DeSimone and Hynes, 1988) at position 799 (Figures 20, 26), however it containss the serine residue characteristic of β3 integrins at position that is lacking in βG.

The βL subunit is observed as a 7.5 Kb maternal transcript and like the βG transcript appears to have long untranslated regions. The βL transcript is maintained at very low levels through to the gastrula stage. In the gastrula there is an increase in the prevalence of the transcript such that it is expressed at high levels through to the pluteus stage of development (Figure 21). This increase in transcription of this subunit is coincident with a time in development when there are large scale cell rearrangements as well as when tissue differentiation is taking place suggesting that this molecule may play a role in these events.

The polyclonal antiserum 73CR raised against the expressed fragment of βL recognizes a protein with an apparent molecular weight of 116 kDa on non-reducing SDS-PAGE. Upon reduction there is the expected increase in relative molecular mass that results from the disruption of the extensive disulphide bonds indicated in the primary sequence of the molecule (Figure 20). Unlike the βG subunit there does not appear to be more than one form of the molecule. While the transcripts for the βL subunit are

detectable at low levels in embryos before the gastrula stage, the antiserum does not detect any protein on western blots. The titre of the 73CR antiserum is much lower than that of the 61CR serum and this may be the reason that the protein is not detected at earlier stages. Both the antisera required extensive schedules of boosting and the expressed fragments do not appear to be highly immunogenic. The high titre found for the 61CR antiserum was obtained after nine consecutive boosts, the 73CR serum was obtained after only five boosts and further immunizations may be required to obtain a higher titre.

The appearance of the βL subunit mimics the transcriptional regulation of the molecule (Figures 21, 22) suggesting the need for the molecule at this time in development is unique and requires *de novo* synthesis. It is interesting that the two β subunits described appear to be regulated transcriptionally since in vertebrates the β subunits are kept as cytoplasmic stores and the transcriptional activation of the molecule does not generally reflect the cytoplasmic pools available for export to the cell surface (DeStrooper *et al.*, 1991; Gawatanka *et al.*, 1992). The coincident expression of transcript and protein may reflect the rapid development that characterizes the sea urchin embryo rather than a novel form of regulation.

4.1 The βL subunit is associated with the SMCs during gastrulation

In situ hybridizations localize the expression of the βL subunit to cells that are at the tip of the extending archenteron (Figure 24). These cells are the presumptive secondary mesenchyme cells as at later stages these cells detach from the tip of the

archenteron and there is no βG activity detected in the remaining archenteron cells. This temporal regulation of expression is rather interesting in that the βL subunit is expressed as long as the cells remain in association with the archenteron, but their presence is not detectable in all the SMCs after that time. This pattern of expression suggests that βL is acting to inhibit the detachment of the SMCs from the archenteron, or that βL is the receptor located in the filopodial extensions that bind to the ECM of the animal hemisphere. The transient attachments that the filopodia make with the ECM may reflect low affinity interactions of the βL receptor with various ligands. Using the same argument, the strong attachments that are observed at the animal pole may be due to a localized ECM component that binds βL with high affinity. Alternatively, the SMCs detach from the archenteron only after the filopodial extensions that these cell send out contact the blastocoelar wall at the animal pole (McClay *et al.*, 1992). Perhaps the SMCs contact the blastocoelar wall and this results in exposure to localized factors that inactivates the receptor and allows the SMCs to detach and migrate freely in the blastocoel. There are examples known where integrins are inactivated after exposure to cytokines (Bartfield *et al.*, 1993). Contact of the PMCs with blastocoelar SMCs is coincident with the inability of these cells to undergo fate conversion suggestive that this contact is mediating the expression of certain genes that assist in defining cell identity (Ettensohn and Ruffins, 1992). Shortly after the SMCs detach from the archenteron the βL subunit is no longer detected by in situ localizations indicating that the βL gene has been transcriptionally inactivated in these cells. Although this has not been documented to be due to contact with other cell populations the timing is coincident with such events.

4.2 The βL subunit is found in pigment cells late in development

The βL transcripts are detectable in a small number of blastocoelar cells that appear to be pigment cells (Figure 24). These cells are primarily located in the vegetal hemisphere and are intermixed with the PMCs during early gastrulation. The location of some of these cells is not characteristic of PMCs suggesting that these are from the pigment cell lineage. At later stages these cells are found throughout the embryo. We do not have the ability to distinguish either PMCs or pigment cells at a morphological level, and while we have markers that are specific for both these lineages, the fixation procedures required for *in situ* localizations prohibit the use of fluorescent probes for localization of cell specific markers. It may be possible to use methods that allow for localization through the precipitation of enzyme substrates, however the markers are cell surface and the washing and extraction steps used during *in situ* localizations may prohibit such experiments. Without distinguishing markers it remains unclear if these cells are derived from the PMC lineage or from the pigment cell lineage. It is interesting that the expression of the βL subunit is detected late in development in the pigment cells, while the βG subunit can be detected at earlier stages of development in these same cells.

5.0 Presumptive roles for the β integrin subunits in sea urchin development

The three sea urchin integrin subunits isolated display a remarkable continuity in their peak periods of expression. The βC subunit expression peaks during cleavage (Figure 12), followed by a peak in expression of the βG subunit during gastrulation

(Figure 15) and a peak in expression of the βL subunit in the pluteus larvae (Figure 21). It is interesting that these peak periods of integrin expression occur during times of morphogenetic movements or expression of ECM molecules in the sea urchin embryo. The timing of the appearance of the βC integrin is coincident with the appearance of hemidesmosomes in the late cleavage stage as well as the compaction of the embryo that occurs as the blastocoel forms (Spiegel and Howard, 1983). The vertebrate β4 integrin is known to be integrated into the mature hemidesmosome and perhaps the βC integrin represents a homologue of this vertebrate molecule. If sequence were available for the cytoplasmic domain of the βC integrin this situation would be easily clarified as the long cytoplasmic domain of β4 is distinctive. Other than a role in the formation of hemidesmosmes or in cell/cell junctions it is difficult to imagine a role for integrins in the pregastrula embryo. At this stage of development there are no cell rearrangements taking place and proper development at this stage is primarily dependant upon cells remaining adherent such that cleavage planes remain correctly orientated.

The βG subunit is expressed maternally and remains at low levels until the late blastula/early gastrula stage of development. At this time the expression levels increase peaking during gastrulation and tailing off into pluteus stage of development. The expression of an integrin at this time is expected as there are a large number of cell rearrangements taking place. The sequences found in the cytoplasmic domain of βG are homologous to those found in the β1 integrins (Figure 26) and are thought to mediate the interaction between the integrin and the cytoskeleton in focal contacts (Lewis and Schwartz, 1995) as well as those that are essential for adhesion to the ECM during

migration (Reszka *et al.*, 1992). The β1 integrins are the receptors that have been implicated most frequently in cell migration suggesting that these conserved sequences play a role in this action. Interestingly the β1 integrins along with a few select β3 integrins are those that bind the ECM molecules containing the RGD sequence as well as other motifs containing the conserved aspartic acid residue (Table 1). Substrates containing these motifs are found in ECM that supports cell migration *in vivo* (Adams and Watt, 1993). The endo 16 molecule is an ECM component that is localized to the vegetal plate and the extending archenteron (Solysikespanola *et al.*, 1994). The coincident expression of an RGD containing substrate and an integrin subunit that contains motifs that are strongly correlated with cell movement is perhaps significant. Collectively, these lines of evidence would indicate that the βG integrins may play a role in cell rearrangements. The high levels of βG that are observed in the pigment cells late in development may indicate that integrins containing the βG subunit are acting in the invasion of the ectoderm. Whereas pigment cells that are still in the blastocoel appear to express βG at high levels, these cells have started to localize at sites where they will eventually invade the ectoderm. βG may play a role in both active migration and in adhesive events that may be static as it is expressed in the pigment cells in the ectoderm. It has been reported that the pigment cells remain motile in the ectoderm (Gibson and Burke, 1985) and perhaps the βG subunit is mediating these events. In the archenteron the expression of βG persists during the time that the archenteron is undergoing active rearrangements yet remains as an epithelium. Without the ability to disrupt the function

of the molecule it is impossible to gain an estimate of the role that the βG integrins play in the archenteron.

The βL subunit would appear to be expressed in the presumptive SMCs while they remain in contact with the archenteron. Receptors containing the βL subunit may mediate the attachment of these cells to an ECM component or to other cells that are in the immediate vicinity. The number of cells that express the βL subunit increases as development proceedes suggesting that as the SMCs undergo division that daughter cells retain expression of the βL subunit. There are relatively few cells in the vegetal plate that express βL (Figure 23), however by the late stages of gastrulation there are a large number of cells expressing the transcript (Figure 24), yet all remain in contact with the archenteron.

In the prism stage embryo the βL subunit begins to appear in what is assumed to be pigment cells (Figure 24). It is uncertain if these cells represent a single population as they have a variety of distinct morphologies. Some of the cells have considerably larger nuclei than others, while others have a distinctive triangular shape and small nuclei. It is difficult to assess if this is due to the shape of the cells or if it is a result of the perspective from which these cells are viewed. However the distribution of the cells strongly suggest that they are pigment cells. If these cells are pigment cells then these cells would appear to express both the βG and βL subunits at the same time. Cells are known to express a large number of distinct integrins at the same time, and these integrins are known to mediate distinct activities (Hynes, 1992). Therefore the coincident expression of βG and βL at the same location is not completely unexpected. The abrupt appearance of these

subunits at the same time is however a bit surprising as presumably they mediate distinct events. Perhaps the situation is similar to that in chick embryos in which cells use at least three integrins that contain the αv subunit for migration. These integrins appear to have distinct functions yet all, and perhaps others, are required for cells to migrate on vitronectin (Delannet *et al.*, 1994).

6.0 Structural and functional considerations

While the three β subunits that have been cloned here likely do not represent the whole spectrum of β integrins that are active during this time of development, it is interesting that all seem to act in static adhesive events. This is not unexpected in that only the β1, β2, and β3 integrins have been implicated in migratory events and the β1 and β3 integrins are also known to mediate static adhesive events. The sea urchin integrins that have been isolated are unique and bear few resemblances to vertebrate integrins making it difficult to correlate primary structure with function. Despite this the βG and βL subunits are most similar to the vertebrate β1 and β3 subunits (Table 2). The cytoplasmic domains of the βG subunit contain sequences that are implicated in cell migration and it is this subunit that is found in the pigment cells during their time of migration. The PMCs also appear to express the βG subunit as they delaminate and migrate into the blastocoel. These cells appear to express the βG subunit up until the time that they stop migrating and arrange in a circular pattern around the vegetal hemisphere. Thus it would appear that this subunit may also be active in cellular movements.

The βL subunit contains a conserved sequence known to be required for the interaction of the cytoplasmic domain with talin, a protein implicated in assembly of the cytoskeleton in focal adhesions. While focal adhesions represent a form of static adhesion found only in cultured cells, the speculated roles of the βL subunit include a number of static adhesive events. The dominant site of early βL expression is in the SMCs at the tip of the archenteron (Figure 24). It is unclear if these cells mediate attachment to the archenteron or adhesion to the ectodermal ECM, however both these events are non dynamic. The βL subunit is also found in pigment cells as they invade the ectoderm, but not as they migrate throughout the blastocoel (Figure 24). Perhaps βL acts in localization of the pigment cells, or the actual invasion of the ectoderm. In all situations it is clear that the βL subunit is expressed during times when cells are not migrating, and this would support a role for this integrin in static events

The βC subunit is the most different from the vertebrate integrins (Figure 26) and it bears approximately equal resemblance to all the vertebrate integrins (Table 2). Due to the sequence divergence in the ligand binding site, it is this molecule which is perhaps the most interesting. Regrettably it is the cytoplasmic region that is most revealing to integrin function and this domain is not included in the clone that was isolated. As described the functional possibilities of βC integrins would appear to be limited due to the temporal regulation of the appearance of the transcript during cleavage. However, the uniqueness of this molecule may reflect it's binding repetoire and if the ligand of this receptor can be found then perhaps primary sequence of this β subunit can be correlated with ligand binding, giving insight into integrin ligand recognition.

It is naive to assume that each of the subunits mediates a single type of adhesive event as many of the vertebrate β subunits form associations with multiple α subunits and the receptors mediate widely varying activities. The speculations discussed above are based upon observations of spatially and temporally restricted expression that is correlated to primary sequence similarities with the vertebrate β integrin subunits. There is expression of the βG subunit in almost all tissues of the embryo during the earlier stages of development (Figure 17). One cannot speculate upon the roles that this molecule plays in these tissues as the expression levels appear to be low but ubiquitous. This would appear to be similar to the expression patterns of the chick β1 (Muschler and Horwitz 1991), and the *Xenopus* β1 subunit (Ransom *et al.*, 1993). Similar to the βG subunit the chick and *Xenopus* subunits are expressed in a general pattern throughout the embryo, and as development proceedes they become progressively localized to specific tissues.

The arrangements of the cysteine residues in the sea urchin subunits merits some discussion as it is both highly conserved and yet divergent in that the placement of one cysteine in each subunit is unique. In all other integrins the arrangement of the cysteines is highly conserved. This conservation in position of cysteine residues extends to the *Drosophila* integrins (Yee and Hynes, 1993). Why the sea urchin integrins do not match this conservation is unknown. The placement of the unique cysteine is similar in all the subunits, however the cysteine that is lost to compensate for this novel location is different in all three sea urchin integrins. The novel location and the distinct site at which a cysteine is lost has implications for the arrangement of cysteine bonds that has been

proposed by Calvete *et al.* (1991) as well as that proposed by Yee and Hynes (1993). None of the sea urchin β integrin subunits can form the disulphide bond arrangements predicted by these authors as the lost cysteines vary in location. It is difficult to speculate on why these changes exist as recent evidence suggests that these cysteine residues are not essential to integrin function (Wippler *et al.*, 1994). The position of the cysteine residues is conserved between *Drosophila* and vertebrates suggesting that the rearrangements observed in the sea urchin molecules are specific to this organism. Perhaps the arrangement of cysteine residues reflects the high salt environment that sea urchins inhabit. Isolation of β integrin subunits from other marine species would assist in clarifying this issue.

7.0 Further considerations

The cell adhesion assays represent a series of preliminary experiments. The results are intriguing, however there are a number of experiments that need to be done to clarify the situation. The antibodies generated against the adherent cells need to be tested against these cells. It is vital to know if the distribution of these epitopes is equal among the attached cells. The antibodies can be used in adhesion assays to evaluate the role that these surface molecules play in cell attachment or fusion. As the attachment and spreading of cells on Pronectin-F appears to be regulated temporally it would be of interest to perform these assays on different stages of development and come to some evaluation as to the temporal distribution of the antibody epitopes.

The βC subunit remains essentially uncharacterized. Isolation of the complete sequence will reveal if this molecule represents a homologue of the vertebrate β4 subunit. In situ localization of this subunit has been started, although these experiments are difficult on early stage embryos. Experiments are underway to raise an antiserum against this molecule. This will allow for an examination of the regulation of translation of this subunit. The production of antisera against expressed proteins has not proven to be especially fruitful in the situations described here. It is unknown why the rabbits respond so poorly to the antigen, and it may be better to express these fragments in eukaryotic cells.

The βG and βL subunits have been partially characterized and it remains inconclusive if the antisera raised against these molecules recognizes epitopes in whole embryos. At the present time immunolocalizations result in very high background and it is difficult to assess the results. It is possible to affinity purify these antisera against the expressed fragments and obtain a cleaner fraction for use in immunolocalizations. The problems associated with these experiments are that the purified serum will be directed against denatured β subunits and this may not localize in embryos. Fixations of the embryos is in itself a problem as previous authors have indicated that integrins must be partially denatured before antisera will recognize the receptor in tissue. These experiments are difficult but feasible.

If clean preparations of antisera can be isolated then immunoprecipitations can be performed. The most obvious experiments that should be done if clean antisera can be prepared is the immunoprecipitation of integrin dimers to get some understanding of the

α subunits associated with the β subunits presented here. These experiments are difficult from a number of standpoints. Labelling of the integrin receptors is not simple, and kinetic labelling does not provide clues to surface expression of molecules. The extraction of integrin receptors from membranes is also difficult as the high salt concentrations that mimic in vivo conditions can be incompatible with extraction buffers. One would have to perform a series of experiments that examine the ability of various buffer and detergent combinations to liberate integrin receptors from isolated membranes. These experiments are further complicated by the observation that the βG and βL subunits appear to be highly sensitive to proteolysis even in the presence of proteinase inhibitors. There are a number of newer proteinase inhibitors available and perhaps some of these will aid in preventing degradation. Again the high salt solutions needed to mimic in vivo conditions make the use of some inhibitors difficult as they precipitate in concentrated salt solutions. If the 61CR and 73CR antisera do immunoprecipitate then they can be used to isolate native protein from denaturing lysates. This protein can then be used to immunize mice and monoclonal antibodies directed against the β subunits screened for their ability to localize or immunoprecipitate dimers. While a long procedure this is perhaps the most productive approach to this problem.

To gain an understanding of the role that these molecules may play in development attempts need to be made to disrupt the function of these molecules. Affinity purified antisera can be injected directly into the blastocoel where it should have easy access to the cells that are expressing the β subunits. The development of inhibitory antibodies directed against integrins has not been predictable, and at this time there are as

many antibodies available that activate integrin function as inhibit it. How the antisera that has been made will perform in such experiments is unpredictable. Alternatively it may also be possible to do some antisense oligonucleotide disruption experiments through the injection of thio-protected oligonucleotides into the blastocoel.

8.0 Summary

In vitro adhesion assays distinguish adhesion events that are specific to integrin receptors. These events appear to be mediated through cells that comprise the epithelial tissues of the mid-gastrula sea urchin embryo, namely the ectoderm and presumptive endoderm. Two complete and one partial β integrin subunit cDNAs have been isolated from the embryos of the sea urchin *Strongylocentrotus purpuratus*. The sea urchin molecules are more closely related to each other than they are to the other known β subunits, and their primary structure deviates from that conserved between all the other known subunits. The isolated cDNAs are expressed at temporal peaks that occur sequentially during development and are coordinated with the major times in development when cell rearrangements are occurring. Two of the isolated β subunits appear to regulate distinct activities. The βG subunit is expressed in actively migrating cells, while the βL subunit is found in cells that are using static adhesive events. The isolation of these molecules provides the tools for the examination of integrin mediated cell adhesion events in a simple model system.

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