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Calcium-binding proteins and development

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The known roles for calcium-binding proteins in developmental signaling pathways are reviewed. Current information on the calcium-binding characteristics of three classes of cell-surface developmental signaling proteins (EGF-domain proteins, cadherins and integrins) is presented together with an overview of the intracellular pathways downstream of these surface receptors. The developmental roles delineated to date for the universal intracellular calcium sensor, calmodulin, and its targets, and for calcium-binding regulators of the cytoskeleton are also reviewed.

Keywords: calcium, EGF-domains, cadherins, integrins, calmodulin, cytoskeleton, Drosophila

Introduction

Although Ca^{2+} has been recognized as an acute regulator of physiological responses for at least a decade, investigation of its possible developmental roles has only recently begun. Ca^{2+} functions in two distinct modes in the developmental processes of multicellular organisms – extracellularly and intracellularly. Outside the cell, several classes of cell-surface receptor molecules with roles in cell-cell, or cell-extracellular matrix, adhesion have proved to be Ca^{2+}-binding proteins with functions in development. Although Ca^{2+} binding has not been shown to be part of the signaling associated with these proteins, the downstream signaling pathways from some of these receptors are amongst the most well-characterized Ca^{2+}-requiring developmental processes known to date. Further, some surface molecules contain novel Ca^{2+}-binding motifs distinct from previously characterized Ca^{2+}-binding domains (see Chapters by Kretsinger, Chazin and Seaton). Inside the cell, although many Ca^{2+}-binding proteins have been identified, their roles in developmental processes are, as yet, very poorly understood or investigated. This deficit has two origins. Firstly, many intracellular Ca^{2+}-binding proteins (for example calpain or annexin family members) have fairly ubiquitous expression patterns and therefore sophisticated genetic methodologies will be required to dissect out their roles in the development of individual tissues. Secondly, methods for imaging intracellular fluxes in Ca^{2+} concentration (Ca^{2+} transients) in intact organisms are only now being developed. Thus identification of the developmental processes that require the action of downstream intracellular Ca^{2+}-binding proteins is in its very early stages. Our review thus focuses on i) the developmental roles of the Ca^{2+}-binding cell surface receptor proteins and their downstream pathways, ii) the roles identified to date for ubiquitous Ca^{2+}-binding proteins in the post-fertilization development of complex, multicellular organisms.

Cell surface, Ca^{2+}-binding, signaling molecules with developmental functions

EGF-domain proteins

This protein family is characterized by extracellular domains that are thought to be involved in protein-protein interactions and that show homology to epidermal growth factor (EGF) (Campbell & Bork 1993). EGF-like domains are 40 to 50 residues in length and contain a conserved set of six cysteine residues that form disulfide bonds in addition to three conserved glycines and one conserved tyrosine residue (Campbell & Bork 1993). Structural analysis has revealed a common structure consisting of two regions of double-stranded antiparallel β sheets, stabilized by disulfide bonds (Figure 1A; Rao *et al.* 1995; Downing *et al.* 1996; Rand *et al.* 1997). Only a subset of EGF-domains can bind Ca^{2+} and these share addi-

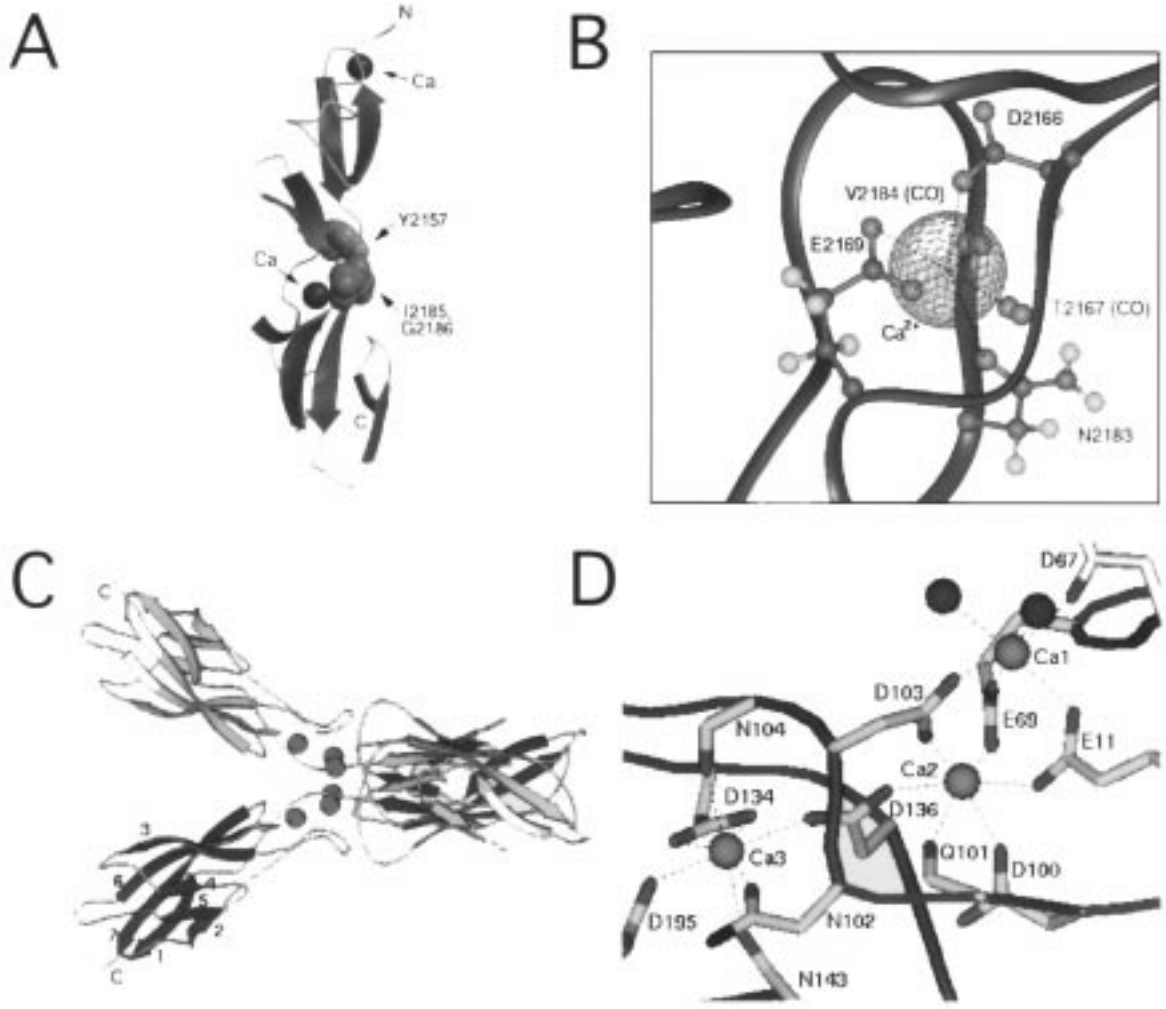

Figure 1. Three dimensional structures of extracellular Ca^{2+}-binding domains.

A. The NMR solution structure of two tandem EGF-like domains from human fibrillin-1. The Ca^{2+}-binding site of the C-terminal repeat is close to the interface between the two domains. Ca^{2+} binding and hydrophobic packing involving the indicated side-chains stabilize the relative arrangement of the two domains.

B. Coordination of Ca^{2+} in the C-terminal repeat of the two fibrillin-1 EGF-like domains shown in A. Six oxygens from the side-chains or backbone carbonyls of the indicated residues coordinate the Ca^{2+}. The seventh ligand required to complete the pentagonal bipyramidal coordination may be provided by an EGF-domain on a neighboring fibrillin-1 molecule.

C. Ca^{2+}-bound homodimer of the two N-terminal repeats of E-cadherin. Interaction in a parallel orientation by the N-terminal repeats from two individual molecules is the basis of the dimerization. The repeats in each monomer have a seven-stranded β-barrel conformation. Three Ca^{2+} ions are bound to the 10-residue linker that connects the two repeats in each monomer.

D. Coordination of Ca^{2+} in the linker region between the two cadherin repeats of each monomer shown in C. Residues and water molecules (unlabeled spheres) that coordinate the bound Ca^{2+} ions (labeled Ca1, Ca2, and Ca3) are shown. Ca1 and Ca2 are each coordinated by seven oxygen atoms from side-chain or backbone carbonyl groups of the residues indicated. Ca3 is coordinated by six oxygen atoms, two of which are contributed by two separate water molecules.

A., C., D. – rendered using Molscript.
B. – rendered using Insight97 (MSI, Inc.).
A., B. – courtesy of Kristina Downing: C., D. – courtesy of Kyoko Yap and Mitsuhiko Ikura.

tional sequence homologies including conserved acidic residues with roles in Ca^{2+} coordination. Interestingly, Ca^{2+} binding is proposed to be an inter-domain phenomenon involving these conserved acidic residues in one EGF-like repeat and a single Ca^{2+} ligand from a neighboring repeat. For one class of repeat pairs, this ligand is thought to derive from the N-terminal adjacent EGF-like domain (Rao *et al.* 1995); for a second class (Figure 1B) liganding in a cross-strand fashion from a repeat on an adjacent molecule has been suggested (Downing *et al.* 1996). Many secreted or transmembrane proteins have multiple, tandemly-arranged extracellular EGF-like domains of both Ca^{2+} binding and non-binding varieties (see Table 1). It is generally believed that binding of Ca^{2+} by EGF-like domains functions to establish and stabilize the relative orientation of the EGF-like domains and thereby determine the overall shape of the molecule. Current evidence suggests that the two classes of repeat pairs showing different Ca^{2+} coordination also differ in the relative orientation of the two tandemly linked repeats (Downing *et al.* 1996).

Ca^{2+}-binding affinities vary between different EGF-like domains even within the same protein and range from 3.1 μM to 9.2 mM, indicating that at extracellular Ca^{2+} levels (around 1.5 mM) some domains would be saturated with Ca^{2+} while others would be only partially saturated (Knott *et al.* 1996; Rand *et al.* 1997). Whether or not local variation in Ca^{2+} concentration is used to affect the structure and hence the function of the Ca^{2+}-binding EGF-domain proteins is unknown at this time.

The Notch/Delta pathway: Of the various developmental roles for proteins with EGF-like domains, those involving the Notch signaling pathway are the best characterized. The *Notch* gene of Drosophila has been extensively studied and is known to regulate cell fate specification in several tissues including the wing, eye, nervous system, and ovary. NOTCH is a large transmembrane protein containing 36 tandem EGF-like repeats in its extracellular domain along with three cysteine-rich LNG (**L**IN-12/**N**otch/**G**LP-1) repeats. Its intracellular domain is characterized by several regions implicated in interactions with cytoplasmic proteins (Kimble & Simpson 1997). NOTCH is a receptor for the extracellular regions of DELTA and SERRATE – two further transmembrane proteins with EGF-like domains containing, respectively, nine and 14 EGF-related repeats (Rebay *et al.* 1991).

Notch signaling is best understood in the specification of neural fate in the Drosophila embryonic peripheral nervous system. Initially, overlapping expression patterns for the proneural genes of the *achaete/scute* complex establish clusters of cells in each embryonic segment that are competent to become neural precursor cells (Campos-Ortega & Jan 1991). In the absence of Notch/Delta signaling all cells of the cluster take on a neural fate, whereas expression of activated NOTCH causes all cells to adopt an epidermal fate (Simpson 1997). NOTCH is thus hypothesized to act in a process termed lateral inhibition (Simpson 1990) that restricts neural fate to one cell in each cluster, with the remaining cells adopting the alternative, epidermal fate. The extracellular domains of NOTCH and DELTA are implicated in initiating the feedback signaling loop that underlies the lateral inhibition process. Both NOTCH and DELTA are initially expressed on the surface of all cells of the proneural cluster; however activation of NOTCH by interaction with DELTA on adjacent cells results in repressing expression of the *achaete/scute* transcription factors which, in turn, directly regulate production of DELTA. Continued signaling through this pathway amplifies small differences in the expression of these genes within a cluster, until eventually expression of *achaete/scute* and DELTA is restricted to a single cell, the neural precursor cell (Simpson 1997).

The repression of *achaete/scute* expression upon NOTCH activation is dependent on the transcription factor *Suppressor of Hairless, Su(H)* (Lecourtois & Schweisguth 1995; Burley & Posakony 1995). The cytoplasmic domain of NOTCH can interact directly with Su(H) protein (Tamura *et al.* 1995) but currently it is unclear whether signal transduction from the plasma membrane to the nucleus involves movement of Su(H) protein or of cleavage products derived from activated NOTCH (Fortini & Artavanis-Tsakonas 1994; Struhl & Adachi 1998). Interestingly, all three elements of the Notch pathway (*Notch*, *Delta* and *Su(H)*) are conserved across all evolutionary orders (Robey 1997; Kimble & Simpson 1997) and in *Caenorhabditis elegans* there is good evidence that these elements are used together as a conserved signaling pathway. Thus, in Caenorhabditis, the NOTCH homologs LIN-12 and GLP-1 are known to be triggered by DELTA homologs such as LAG-2, leading to activation of transcription factors (LAG-1 and CBF-1) homologous to Su(H) protein (Kimble & Simpson 1997).

In Xenopus, a similar mechanism of lateral inhibition is at work in the selection of neural cells during embryogenesis and Notch/Delta signaling also appears to be important for proper development of the retina (Robey 1997). In mammals, the

Table 1. Proteins with EGF-like domains that have known developmental roles

Protein	Species	Developmental roles	EGF-like domains*	Other domains	References
LNG receptor family (LIN-12/NOTCH/GLP-1)	Caenorhabditis (*glp-1*, *lin-12*) Drosophila (*Notch*) vertebrates	multiple lateral and inductive cell fate determinations, especially neuronal vs. epidermal	10, 13 36 (21) variable	LNG(3), cd10(6), TM	Kimble & Simpson 1997 Robey 1997 Young & Wesley 1997
DSL ligand family (DELTA/SERRATE/ LAG-2)	Caenorhabditis (*Lag-2*, *Apx*-1) Drosophila (*Delta*, *Serrate*) vertebrates	see above; ligands for LNG receptors	1, 4 10, 14 variable	TM	Gao and Kimble 1995 Lindsell *et al*. 1995 Dornseifer *et al*. 1997 Robey 1997
Netrins	C. elegans (*unc6*) Drosophila (*Netrin-A*, *Netrin*-B) vertebrates (*Netrin-1, Netrin-2*)	neuronal guidance	3		Serafini *et al*. 1994 Harris *et al*. 1996 Wadsworth *et al*. 1996
CRUMBS	Drosophila	epithelial cell polarity	30	LNS (4), TM	Grawe *et al*. 1996 Knust *et al*. 1993
SLIT	Drosophila	midline glia, commissural axons	7	LRR (4), LNS	Rothberg *et al*. 1990 Zhou *et al.* 1997
FAT	Drosophila vertebrates	tumor suppressor	5	LNS, cadherin (34), TM	Agrawal *et al*. 1995 Dunne *et al*. 1995
BMP1	vertebrates sea urchin Drosophila (*tolloid*)	limb development, dorsal-ventral axis formation	2(2)	metallo-proteinase, Clr/s(5)	Goodman *et al*. 1998 Fukagawa *et al*. 1994 Finelli *et al*. 1994
tenascin	vertebrates	neurite extension	variable		Mackie 1997 Taylor *et al*. 1993
fibrillin	human	connective tissue (Marfan's syndrome)	47(43)		Wu *et al*. 1995
neurexin	vertebrate	neuronal cell recognition	3	LNS (6), TM	Missler & Sudhof 1998
CASPR	rat Drosophila (*neurexin IV*)	neuronal-glial interactions, septate junctions	2	LNS (4), TM	Missler & Sudhof 1998 Baumgarter *et al*. 1996

*number of Ca^{2+}-binding EGF-like domains is indicated in parenthesis where known.
cdc10–cdc10/ankyrin repeat, LRR-leucine rich repeat, LNS-laminin A/neurexin/sex hormone-binding globulin repeat, TM-transmembrane domain.

widespread expression patterns of the known NOTCH homologs suggests that they play numerous roles in development, with strong evidence that a Notch pathway regulates multiple cell fate decisions in T-cell development (Robey 1997).

Lateral inhibition is not the only signaling mode in which NOTCH operates in these various developmental situations, however. In Drosophila and other organisms, NOTCH-mediated inductive signaling and interactions between the Notch pathway and other signaling pathways have been identified (Simpson 1997). Further, some roles of Notch have been shown genetically be independent of Su(H), indicating that other downstream effector proteins remain to be identified (Matsuno *et al.* 1997).

Several studies have explored the function of EGF-like repeats in NOTCH/DELTA interactions. Two of the 36 EGF domains (#s 11 and 12) of Drosophila NOTCH have been shown to be necessary and sufficient for Ca^{2+}-dependent interaction with either DELTA and SERRATE in cell aggregation assays (Fehon *et al.* 1990; Rebay *et al.* 1991). However, *in vivo*, changes to certain EGF-like domains of DELTA can suppress the phenotypic effects of mutations to NOTCH EGF-like repeats other than #s 11 or 12 without restoring wild-type binding between the NOTCH and DELTA protein pair, as assayed by cell aggregation (Leiber *et al.* 1992). These results suggest that the mechanisms of interaction between NOTCH and DELTA, and presumably other ligands, varies in different developmental situations.

Other EGF-like domain proteins with developmental roles: Other EGF-like domain proteins are involved in developmental processes and are listed in Table 1. For most of these proteins, the specific role of Ca^{2+}-binding EGF-domains has not been addressed, however it is assumed that these domains are used in protein-protein or protein-matrix interactions. Many other EGF-domain proteins exist for which no developmental role has been shown. This is largely because most of these proteins have been studied in non-developmental systems such as tissue culture cells. Systematic study of EGF-like domains in development may reveal roles for many more of the proteins that bind extracellular Ca^{2+}.

Cadherins

The cadherins are a family of integral membrane glycoproteins whose large, Ca^{2+}-binding, extracellular domains promote homophilic cell-cell interactions. Disruption of cadherin-based cell-cell interactions is the likely basis of many early observations on the Ca^{2+}-dependence of cell-cell adhesion and selective reaggregation (reviewed in Geiger & Ayalon 1992). Since the first purification of a fragment of E-cadherin (epithelial-cadherin) by Hyafil *et al.* (1980), a large family of related proteins has emerged (Table 2), with currently approximately 30 members identified. Cadherins are found in both vertebrates and invertebrates and are expressed by most cell types of multicellular organisms (reviewed in Aberle *et al.* 1996). In addition to mediating Ca^{2+}-dependent homophilic cell-cell adhesion, cadherins are essential components of adherens junctions and desmosomes. Structurally all cadherins have a large extracellular domain, a single transmembrane domain, and a modest intracellular domain, usually of less than 200 amino acids (Nagafuchi *et al.* 1987). Typically the extracellular domain is composed of five tandem copies of a unit termed the cadherin repeat. Each repeat is about 110 amino acids long. Structural data (Overduin *et al.* 1995; Nagar *et al.* 1996) for the Ca^{2+}-bound form of the two N-terminal repeats of E-cadherin (outermost with respect to the plasma membrane) and the N-terminal repeat of neural (N-) cadherin (Shapiro *et al.* 1995) have provided insight into several aspects of cadherin function. Thus, several novel sequence motifs (PENE, LDRE, DQNDN, and DAD) proved, as predicted, to play a role coordinating Ca^{2+} ions (three total) at the junction between E-cadherin repeats 1 and 2 (Figure 1C, D) and, based on the conservation of these sequences throughout the extracellular domain, it is proposed that a total of 12 Ca^{2+} ions per molecule are bound at the four junction regions between cadherin repeats in the extracellular domain. The functional unit of the cadherins is a homodimer and the structural studies demonstrate that this is a parallel homodimer (Figure 1C), with interactions between the N-terminal repeat in each subunit contributing strongly, if not exclusively, to dimer formation. This N-terminal repeat is also the site of the adhesion surface and conserved HAV motif required for interaction with cadherins on other cells, an interaction proposed by Shapiro *et al.* (1995) to involve antiparallel repeat binding. The binding of Ca^{2+} ions is essential for the adhesive function of cadherins (Takeichi 1990) and the study of Nagar *et al.* (1996) reveals that Ca^{2+} binding has at least two roles; it generates linearity and rigidity in the extracellular domain and it generates and stabilizes the surfaces critical for homo-dimer formation and cell-cell interaction.

Intracellularly, cadherins are complexed with β-catenins or plakoglobins (γ-catenins) (reviewed in

Table 2. Cadherins and their phylogenetic and tissue distributions

Cadherin type	Tissue[a]	References
E-cadherin (type I)	Various vertebrates and invertebrates: early embryo, epithelium, ovaries, kidney, placenta	b
N-cadherin (type I)	Various vertebrates and invertebrates: lens, early embryo, nerves, muscle, ear endothelium	b b
P-cadherin (type I)	Mouse: placenta, skin Human: epithelium Bovine: endothelium	
R-cadherin (type I)	Chicken: retina	b
EP-cadherin (type I)	Xenopus: early embryo	b
B-cadherin (type I)	Chicken: early embryo	b
M-cadherin (type I)	Mouse: muscle	b
Type II cadherin (cadherin 5-12)	Human and mouse	Reviewed by Takeichi 1994
T-cadherin (truncated cytoplasmic domain)	Chicken: early embryo	b
Desmogleins		b
Desmocollins		b
Protocadherins	Vertebrate and invertebrate central nervous system	Sano *et al.* 1993
fat gene product and *dachsous* gene product	Drosophila: imaginal discs	Mahoney *et al.* 1991 Clark *et al.* 1995
ret gene product	Human and mouse	Iwamoto *et al.* 1993

Modified from Geiger and Ayalon (1992),
[a]Information not available for all cadherins.
[b]: Geiger & Ayalon 1992.

Aberle *et al.* 1996). These proteins bind to α-catenin, which links the cadherin-catenin complexes to the actin cytoskeleton. It is through the interaction with β-catenin that cadherins are implicated in developmental processes. Thus, surprisingly, studies mainly conducted in Drosophila and Xenopus, have shown that β-catenin is a component the Wg/Wnt developmental signaling pathway (Peifer 1995; Funayama *et al.* 1995). In Drosophila, where the pathway is best characterized, Wg/Wnt has been shown to act at multiple points in development. It is not only involved in segment polarity determination and head formation during embryogenesis but also in anterior-posterior and dorsal-ventral axis patterning in the imaginal discs. The essential molecular components of the Wnt pathway have been identified by genetic and other analyses (reviewed in Nusse 1997). Binding of the secreted Wg/Wnt ligand to its receptor (a member of the *frizzled* family) at the cell surface activates a transcriptional role for the Drosophila β-catenin homolog, ARMADILLO (ARM). Presumably the transcriptional targets regulated as a result of ARM/β-catenin activity execute the pattern formation and developmental roles of Wg/Wnt. The transcriptional role of ARM/β-catenin involves association with a DNA binding protein of the Lef-1/TCF (**T** **C**ell **F**actor) family. The exact mechanism of activation of ARM/β-catenin is not yet clear, but appears to involve the release of free β-catenin from a complex containing the *Zw-3* (*Zeste-white* 3)

Table 3. Integrins and their ligands

Integrin type[a]	Name	Ligands
β1 integrins	$\alpha_1\beta_1$ (VLA-1)	collagen (I, IV), laminin
	$\alpha_2\beta_1$ (VLA-2)	collagen (I-VI), laminin, fibronectin
	$\alpha_3\beta_1$ (VLA-3)	epiligrin, laminin, nidogen/entactin, fibronectin, collagen I
	$\alpha_4\beta_1$ (VLA-4)	[g]fibronectin.alt, VCAM-1/INCAM110
	$\alpha_5\beta_1$ (VLA-5)	fibronectin
	$\alpha_6\beta_1$ (VLA-6)	laminin
	$\alpha_7\beta_1$ (VLA-7)	laminin
	$\alpha_8\beta_{1b}$ (VLA-1)	tenascin, fibronectin, vitronectin
	$\alpha_9\beta_{1c}$ (VLA-1)	tenascin
	$\alpha_v\beta_1$ (VLA-1)	fibronectin
β2 integrins	$\alpha_1\beta_2$ (LFA-1)	ICAM-1 to 3
	$\alpha_m\beta_2$ (Mac-1)	C3bi, factor X, fibrinogen, ICAM-1
	$\alpha_x\beta_2$ (p150, 95)	fibrinogen, C3bi
β3 integrins	$\alpha_{IIb}\beta3$ (GPIIb-IIIa)	fibrinogen, fibronectin, von Willebrand's factor, vitronectin
	$\alpha_v\beta3$ (VNR)	fibrinogen, fibronectin, von Willebrand's factor, vitronectin, thrombospondin, osteopontin, bone sialoprotein 1
β7 integrins	$\alpha_{IEL}\beta7$ (M290IEL)	unknown
	$\alpha_4\beta7$ (LPAM-1)	[g]fibronectin.alt, VCAM-1
β4 integrins	$\alpha_6\beta4$	laminin
β5 integrins	$\alpha_v\beta5$	vitronectin
β6 integrins	$\alpha_v\beta6$	fibronectin
β8 integrins	$\alpha_v\beta8$	unknown
Drosophila PS integrins[f]	PS1[d] ($\alpha_{PS1}/\beta PS$)	laminin
	PS2[d] ($\alpha_{PS2}/\beta PS$)	tiggrin
	PS3[e] ($\alpha_{PS3}/\beta PS$)	laminin A

Modified from Sonnenberg 1993.
[a]All found in vertebrates unless specified.
Other references: [b]Schnapp *et al*. 1995, [c]Weinacker *et al*. 1995, [d]Gotwals *et al.* 1994, [e]Stark *et al*. 1997.
[f]β_{PS} is encoded by *myospheroid;* α_{PS1} is encoded by *multiple edematous wings*; α_{PS2} is encoded by *inflated*; and α_{PS3} is encoded by *scab*.
[g]fibronectin containing the IIICS integrin-binding region.

protein kinase and the tumor suppressor protein APC (**A**denomatous **P**olyposis **C**oli). Free cytoplasmic β-catenin then associates with Lef-1/TCF and translocates to the nucleus. Although no active role for the cadherin-β-catenin interaction in developmental processes has yet been identified, it is tempting to speculate that there is a dynamic balance between the cadherin-associated β-catenin pool, the *Zw3*-APC-associated pool and the Lef-1/TCF-associated pool that is regulated both by the Wg/Wnt ligand-receptor interactions and by cadherin-mediated cell-cell adhesions. Such an interaction could explain why cellular behavior such as differentiation is affected by cadherin-mediated cell-cell adhesions.

Integrins

In addition to cell-cell interactions, adhesive interactions between cells and the extracellular matrix (ECM) are critical to cell migration, proliferation and differentiation. These cell-ECM interactions are frequently mediated by the integrins, another large family of cell surface glycoproteins with members that show specificity for interaction with particular ECM components such as fibronectin or vitronectin (reviewed in Hynes 1987). More than 20 family members are known to date (Sonnenberg 1993; Tozer *et al.* 1996). Although they show differing tissue-specific expression patterns, virtually every known cell type expresses at least one, and often

several, type of integrin subclass. All integrins consist of an α subunit and a β subunit and are divided into subfamilies based on their β subunit type. Table 3 lists these subfamilies and their specific extracellular matrix protein ligands.

Both integrin subunits contain a large extracellular domain of about 750 (β subunit) or 1000 (α subunit) residues, a single transmembrane domain, and a short cytoplasmic domain typically of less than 65 residues. Four motifs related to the EF-hand class of Ca^{2+} binding sites (see Chapter by Kretsinger) are found in the extracellular domain of a typical α subunit and these sequences are thought to be responsible for the divalent cation binding properties of the subunit (Sonnenberg 1993, Tozer *et al.* 1996). Although Ca^{2+}, Mg^{2+} and Mn^{2+} all bind to integrins, in some cases these various cations produce different effects on a given integrin or a given cation produces differing effects on different integrin types. Thus, it is presently unclear for any integrin which cation is the natural ligand at any of its four putative binding sites. Some α subunits contain only three EF-hand-like motifs and an additional extracellular 'I domain'. High resolution structures for the Mg^{2+} and Mn^{2+} forms of this domain have revealed that it contains a modified EF-hand-like motif in which cation coordination involves non-contiguous residues in the protein (Lee *et al.* 1995). Mutagenesis and modeling studies suggest that an 'I domain'-like region of the β subunit also binds cations (Tozer *et al.* 1996).

Ca^{2+}/divalent cation binding is essential for optimal interaction of integrins with their extracellular ligands and available data suggest the interesting possibility that some of the bound cations, particularly the cation occupying the I-domain, are an integral part of an integrin-ligand complex (Tozer *et al.* 1996). Interestingly, the interaction between integrin $\alpha_v\beta_3$ and its ligand osteopontin is exceptional in that adhesion between the two proteins diminishes with increasing Ca^{2+} levels (Hu *et al.* 1995). As a result, osteoclasts (which express $\alpha_v\beta_3$) show diminished binding to the extracellular matrix of bone at high Ca^{2+}, a mechanism which prevents unnecessary bone reabsorption. However, Ca^{2+}-binding to osteopontin itself (Chen *et al.* 1992), as opposed to integrin $\alpha_v\beta_3$, may be the cause of this unusual interaction.

The adhesion roles of integrins appear to be mediated via binding of their cytoplasmic domains to proteins such as talin and α-actinin which interact with the actin cytoskeleton (Plopper & Ingber 1993). Recently however, integrins have been recognized as simultaneously mediating both adhesion and 'outside-in' signal transduction to regulate cytoskeletal structure and overall cell behavior (for reviews, Sjaastad & Nelson 1997; Brakebusch *et al.* 1997). Several intracellular signaling molecules are known to be activated in response to integrin-ECM interaction. Kinases such as focal adhesion kinase (Schaller *et al.* 1995), integrin-linked kinase (Dedhar & Hannigan 1996), protein kinase C and tyrosine kinases are upregulated, as is a pathway involving rho (reviewed in Clark & Brugge 1995). However, no complete signal pathway has been fully delineated for any integrin-linked response to date. Most excitingly, in the context of this review, integrin ligation frequently results in transient increases in intracellular Ca^{2+} levels. The mechanism for coupling integrin ligation to initiation of these Ca^{2+} transients is not fully understood, and may involve different sources for the Ca^{2+} (extracellular as opposed to intracellular) for different integrin-ECM interactions. There is evidence however that these Ca^{2+} transients mediate intracellular changes that feed back to the integrins, as part of 'inside-out' signaling, to modify their adhesive roles (Sjaatstad & Nelson, 1997). Interestingly, the Ca^{2+}-binding protein calreticulin, previously viewed largely as a high capacity Ca^{2+} buffer of the luminal Ca^{2+} stores, has recently been shown to associate with the cytoplasmic tails of integrins and to be essential for the Ca^{2+} transients induced on integrin-ECM interaction (Coppolino *et al.* 1997).

Given that integrins mediate complex cell behavior in response to the ECM, it seems clear that they will play critical roles in many developmental processes. Convincing evidence for their developmental functions has come from mutational studies in Drosophila. The Drosophila genome encodes three α and one β integrin subunit (Table 2) and mutations have been generated for all four genes (Gotwals *et al.* 1994). The β subunit associates with each of the three α subunits to form three different complexes termed **P**osition **S**pecific integrins, PS1, PS2 and PS3. This nomenclature reflects the position-specific expression in the third instar wing imaginal discs of PS1 and PS2.

The phenotypes of alleles of each of the four integrin genes have been studied in embryos, wing imaginal discs, and developing eyes (Brown 1994; Brower *et al.* 1995; Stark *et al.* 1997). Null mutations of the common β subunit gene *myospheroid* (*mys*) cause embryonic lethality, with defects in the attachment of the somatic and visceral muscles to the epidermis and midgut epithelium. Further, although appropriate epithelial cell-shape changes are executed to initially produce dorsal closure (the joining of two

epithelial sheets on the dorsal midline), mutant embryos fail to maintain dorsal closure. Mutant abnormalities seen at later stages include defective attachments between the epithelial cells of the developing dorsal and ventral wing surfaces and detachment of the developing retinal epithelium from its underlying basement membrane. The defects seen for null mutations of the genes encoding the α subunits of PS1 (*multiple edematous wings, mew*), PS2 (*inflated*, *if*) or PS3 (*scab*, *scb*) correspond to subsets of the phenotypic defects seen for mutations in their common β subunit.

SPARC

The extracellular protein SPARC (**S**ecreted **P**rotein **A**cidic and **R**ich in **C**ysteine, also called BM-40 and osteonectin) binds two Ca^{2+} ions per molecule via EF-hand type binding motifs (Hohenester *et al.* 1997). While the most studied aspects of SPARC function involve its roles as an anti-adhesive and anti-proliferative protein in tissue repair and remodeling (Goldblum *et al.* 1994, Jendraschak & Sage 1996), a few studies suggest developmental roles for SPARC. Disruption of SPARC function causes defects in branching morphogenesis during rat lung development (Strandjord *et al.* 1995). Also, ectopic expression of SPARC in Xenopus causes severe dorso-ventral axis defects (Damjanovski *et al.* 1997) and in Caenorhabditis results in embryos with defects in vulva and muscle formation (Schwarzbauer & Spencer 1993). All of these functions are dependent upon Ca^{2+} binding by the EF-hand sites of SPARC.

Developmental roles for intracellular Ca^{2+}-binding proteins

Calmodulin and its targets

Calmodulin (Cam) is one of the most ubiquitous mediators of intracellular Ca^{2+} signaling and it is reasonable to assume that developmental processes involving Ca^{2+} transients will involve Cam-mediated steps. Although Drosophila offers the most potential for identifying Cam-regulated developmental steps via genetic routes, the roles of Cam in embryogenesis cannot be studied by a simple genetic approach in this organism since maternally-derived Cam protein is used to support embryonic development (Heiman *et al.* 1996). Thus *Cam* null mutant animals proceed through embryogenesis normally and die soon after hatching with physiological, as opposed to developmental, defects (Heiman *et al.* 1996). However, through the use of constructs that specifically disrupt Cam signaling in the growth cone of developing neurons, VanBerkum and Goodman (1995) have demonstrated a role for Cam in axon guidance in developing neurons of the embryonic CNS. Phenotypic analysis of mutations that alter the amino acid sequence of Cam in Drosophila (as opposed to completely eliminating the protein) is also providing useful insight into the later developmental roles of Ca^{2+}-Cam signaling (Nelson *et al.* 1997). Most strikingly, a single amino acid change produces a failure of head eversion during the developmental reorganization events of the pupal stage and individuals die during this period with the adult head internalised in the upper body cavity. This failure reflects loss of a developmentally regulated series of muscle contractions that expel the head, indicating defective interaction with a muscle-specific Cam target.

In mammalian systems, given the presence of three genes encoding Cam in the genome (Nojima 1989), over-expression of Cam and use of constructs to inhibit Cam action have been the only routes used thus far to address Cam's roles in developmental processes. Directed expression of an inhibitor of Cam signaling has identified a role for Cam in the development of type II epithelial cells of the lung and demonstrated the critical role of these cells in the branching morphogenesis of the bronchial tree (Wang *et al.* 1996). Pronounced hypertrophy induced by cardiac overexpression of Cam provided the first indication that Cam plays a role in mammalian heart development (Gruver *et al.* 1993). More recently, transcriptional-activation induced by the Cam target calcineurin, a protein phosphatase, has been shown to underlie this hypertrophic response (Molkentin *et al.* 1998). Calcineurin dephosphorylates the transcription factor NF-AT3, resulting in NF-AT3 movement into the nucleus and interaction with the factor GATA4. Even more intriguingly, mutation of another member of the NF-AT family ($NF\text{-}AT_c$) that also appears to require calcineurin activation, has been shown to disrupt valve and septum formation in the developing heart (De la Pompa *et al.* 1998; Ranger *et al.* 1998). Multiple transcriptionally mediated roles for calcineurin in heart development are thus indicated.

In *Xenopus*, evidence that calcineurin plays a role in dorsal-ventral axis formation during early embryonic patterning has been presented (Nishinakamura *et al.* 1997). Similarly, the demonstration that Ca^{2+} induces dephosphorylation of DORSAL, the key transcriptional regulator in dorsal-ventral patterning in Drosophila embryos, has been interpreted as indi-

cating a role for calcineurin in dorsal-ventral axis formation in this organism too (Kubota & Gay 1995).

In contrast to protein dephosphorylation by calcineurin, Ca^{2+}-Cam regulation of developmental processes by activation of target protein kinases is largely unexplored at this time. However, the induction of Cam kinase IV in developing rat brain neurons by thyroid hormone (Krebs *et al.* 1996) and its ability to phosphorylate the transcription factor CREB (Deisseroth *et al.* 1998) suggest strongly that Cam kinase IV will have roles in developmentally regulated gene expression.

Three highly-related multi-domain proteins from rat, Caenorhabditis and Drosophila that contain a Cam kinase-like domain, complete with Cam-binding region, have recently been identified (Dimitratos *et al.* 1997). Interestingly the Caenohabditis homolog is the protein encoded by *lin-2*, a gene that is part of an EGF receptor /Ras pathway required for vulval development. Other domains in these three proteins define them as a new subfamily of the MAGUKs (**M**embrane-**A**ssociated **GU**anylate **K**inase homologs), proteins that are involved in sub-plasma membrane and cell-junctional functions including developmental cell-cell signaling events. Although the work with *lin-2* indicates that the Cam kinase domain is not active as a kinase (Hoskins *et al.* 1996), it remains to be determined how important Cam binding to this domain is to the developmental role of the protein.

The sub-plasma membrane cortical cytoplasm contains a number of other Cam-binding cytoskeleton-related proteins and several of these have been implicated in effecting morphogenetic changes during development as a result of mutational studies in Drosophila. The Drosophila homologue of protein 4.1 (*coracle*) appears to have a more specialized role than its mammalian counterpart. The Cam-binding region is well-conserved but sequences implicated in interaction with actin are missing and CORACLE is concentrated at septate junctions in epithelial cells as opposed to being widely dispersed along the inner side of the plasma membrane (Fehon *et al.* 1994). Strong *coracle* mutations produce embryonic death as a result of failure in dorsal closure. Although this phenotype could simply reflect an 'effector' role for CORACLE in dorsal closure, genetic interactions between *coracle* alleles and a dominant mutation of the EGF-receptor homologue suggest that CORACLE may play a subtler role involving cell-cell signaling associated with morphogenetic movements.

For α-spectrin, a membrane cytoskeletal protein with both Cam- and EF-hand Ca^{2+}-binding domains, a maternal supply of protein delays the expression of mutant phenotypes until the larval stages. The major mutant defects seen prior to death in these stages involve the cuprophilic cells of the midgut and include deranged cell contacts and failure of acid secretion by these cells (Lee *et al.* 1993; Dubreuil *et al.* 1998). These defects appear to reflect loss of both organization within the cortical cytoplasm and proper formation of specialized subdomains of the plasma membrane – processes previously indicated to require spectrin function. Interestingly the phenotype of a mutation that generates α-spectrin missing its second EF-hand Ca^{2+}-binding site suggests a role for this site in membrane association of the protein (Lee *et al.* 1993).

During oogenesis in Drosophila, an unusual series of incomplete cell divisions generates a syncytium of 16 interconnected cells, one of which will become the oocyte proper. A structure termed the fusome links these cells (cystocytes) in a precise pattern. Both α-spectrin and the Drosophila homolog (ADD-HTS) of a further Cam-binding cortical cytoskeleton protein, adducin, are major components of the fusome (Lin *et al.* 1994). Mutational analysis has shown that in the absence of either protein, the fusome is defective, the number of cystocyte divisions is reduced and determination of one cell as the oocyte frequently fails (Yue & Spradling 1992; De Cuevas *et al.* 1996). If fusome formation is considered a specialized use of more generalized cellular functions, these findings suggest a general role for membrane associated Ca^{2+}-regulated cytoskeletal proteins in controlling both cell division and sister cell specialization after developmentally significant mitoses. Whether Ca^{2+}-Cam regulation of ADD-HTS function is important in these processes in Drosophila is unclear however, since at least four isoforms of the ADD-HTS protein are present in the developing oocyte (Zaccai & Lipshitz 1996) and at least one of these lacks the Cam-binding region (Yue & Spradling 1992).

The effects of loss of α-spectrin in the somatic follicle cells surrounding the developing oocyte (Lee *et al.* 1997) and of loss of ADD-HTS during early embryogenesis in Drosophila (Zaccai & Lipshitz 1996) suggest further widespread roles for membrane cytoskeletal organisational events in developmentally regulated cell and nuclear divisions.

The Ca^{2+}-release channels of the smooth endoplasmic reticulum, the inositol (1, 4, 5) tri-phosphate (IP3) and ryanodine (RyR) receptors, are both thought to be both Ca^{2+}- and Cam-binding proteins although the binding sites for neither entity

have yet been defined on either native protein. Whereas mutant studies of the RyR receptor in Caenorhabditis suggest purely physiological roles for the channel in regulation of body wall muscle contraction (Maryon *et al.* 1996), for the IP3 receptor, genetic studies in both Caenorhabditis and Drosophila are beginning to identify developmental processes in which these channels play a role. A major defect seen in IP3 receptor mutant Drosophila larvae is failure to moult as a result of reduced levels of ecdysone, the hormone which orchestrates developmental progression through the larval stages (Venkatesh & Hasan 1997). A role for the IP3 receptor in the pathways involved in ecdysone release and synthesis has thus been proposed. In addition to moulting failures, IP3 receptor mutant larvae also show widespread and dramatic defects in cell growth, proliferation and differentiation (Acharya *et al.* 1997) suggesting fundamental roles in many aspects of development. Strikingly, in Caenorhabditis, the IP3 receptor and IP3 kinase have both proven to specifically mediate one of the five developmental roles of the EGF receptor – that of dilation of the spermathecae in hermaphroditic animals (Clandinin *et al.* 1998). This role is interesting in that it adds to the growing number of observations that indicate a role for intracellular Ca^{2+} release in muscle relaxation as opposed to contraction.

Ca^{2+}-binding proteins and the cytoskeleton

One function imputed to intracellular increases in Ca^{2+} is remodeling of the cytoskeleton. Members of the gelsolin/villin family of actin-associated proteins represent the best characterized potential effectors of this function since these proteins are known to cap, nucleate, bundle or sever actin fibers in a Ca^{2+}-dependent manner (Weeds & Maciver 1993). Preliminary evidence indicates that Ca^{2+} binding to gelsolin involves binary complex formation with actin (Weeds & Maciver 1993). As for many of the protein families described here, the functions of gelsolin/villin isoforms have largely not been examined in developmental situations. However, developmental roles for two family members have been identified in Drosophila through genetic analysis. Mutations to a gelsolin homolog (*flightless-1*) produce severe disorganization of flight muscle structure and also defects in the cell shape changes and movements associated with gastrulation (Campbell *et al.* 1993). A germ-line specific villin homolog (*quail*) has proved essential for the actin bundling events associated with bulk transfer of cytoplasm from the syncytially linked sister cells of the oocyte into the oocyte proper (Mahajan-Miklos & Cooley 1994).

Conclusions

In this review, we have addressed the extracellular and intracellular roles of Ca^{2+} in developmental processes. Currently, most evidence supports the view that Ca^{2+} binding to protein domains outside the cell largely plays a structural role, generating the conformations required for cell-cell and cell-matrix interactions. However, the possibility of a more dynamic role, with Ca^{2+} acting to signal environmental changes via binding to some of these extracellular domains, still remains open.

Inside the cell, developmental defects have been demonstrated for mutations in a number of Ca^{2+}-binding proteins. However, the delineation of developmental pathways initiated by Ca^{2+} transients that lead to activation of key Ca^{2+}-binding proteins is essentially unexplored. Currently, the role of calcineurin in cardiac hypertrophy (see above) is perhaps the best described such pathway although even in this case, an initiating role for Ca^{2+} has not been demonstrated. As new Green Fluorescent Protein-based Ca^{2+} sensors (Persechini *et al.* 1997; Miyawaki *et al.* 1997) are put to use to investigate the roles of Ca^{2+} in whole organisms, we can hope to see Ca^{2+} regulation of developmental processes finally explored in detail.

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