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The role of cadherin cell adhesion molecule in the early embryonic development of Xenopus laevis

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THE ROLE OF CADHERIN CELL ADHESION MOLECULE IN THE EARLY EMBRYONIC DEVELOPMENT OF XENOPUS LAEVIS

by

YOUNG-SOOK CHOI

DISSERTATION

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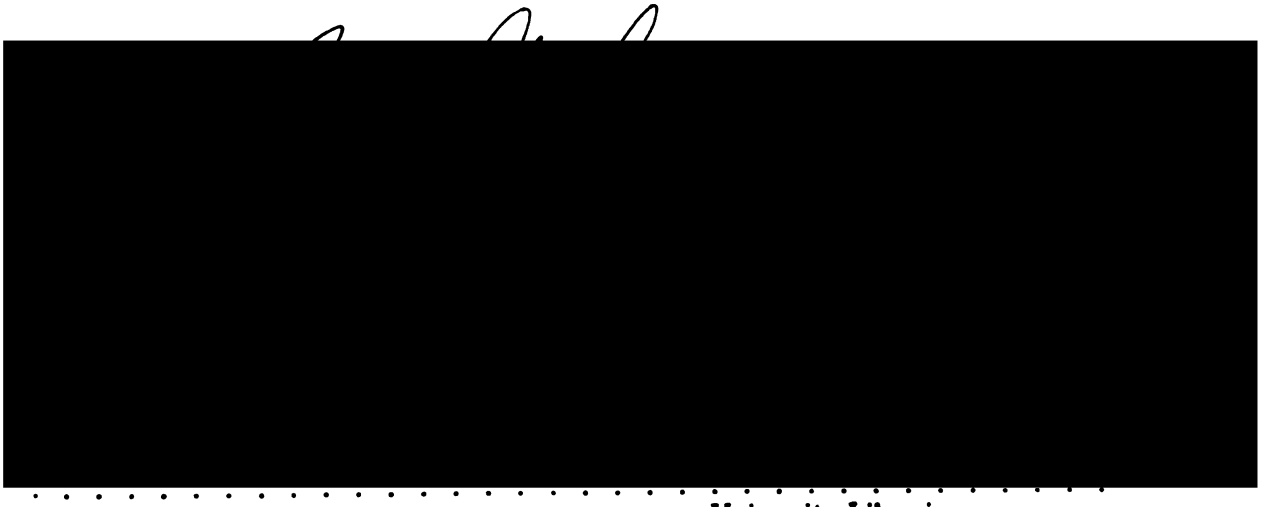

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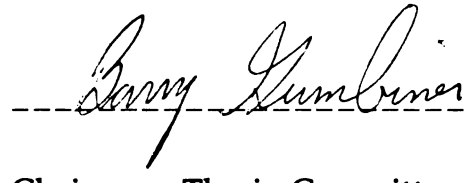
THE ROLE OF CADHERIN CELL ADHESION MOLECULE IN THE EARLY EMBRYONIC DEVELOPMENT OF XENOPUS LAEVIS.

Young-Sook Choi

Ph.D. Pharmacology



Chairman, Thesis Committe

Dissertation Abstract

E-cadherin was identified in the *Xenopus* A6 epithelial cell line by antibody cross-reactivity and several biochemical characteristics. Four independent mAbs generated against purified *Xenopus* E-cadherin recognized the same polypeptides in A6 cells, adult epithelial tissues, and embryos. These mAbs inhibited the formation of cell contacts between A6 cells and stained the basolateral plasma membranes of A6 cells, hepatocytes, and alveolar epithelial cells.

The time of E-cadherin expression in early *Xenopus* embryos was determined by immunoblotting. Unlike its expression in early mouse embryos, E-cadherin was not present in the eggs or early blastula of *Xenopus laevis*. These findings indicate that a different Ca^{2+}-dependent cell adhesion molecule, perhaps another member of the

cadherin gene family, is responsible for the Ca^{2+}-dependent adhesion between cleavage stage *Xenopus* blastomeres. Detectable accummulation of E-cadherin started just prior to gastrulation at stage 9 1/2, and increased rapidly up to the end of gastrulation at stage 15. In stage 15 embryos, specific immunofluorescence staining of E-cadherin was discernible only in the ectoderm, but not in the mesoderm and the endoderm. The ectoderm at this stage consists of two cell layers. The outer cell layer of the ectoderm was stained intensely and staining was localized to the basolateral plasma membrane of these cells. Lower levels of staining was observed in the inner cell layer of the ectoderm. The coincidence of E-cadherin expression with the process of gastrulation and its predominance in the ectoderm suggest that it may play a role in the morphogenetic movements of gastrulation and resulting segregation of embryonic germ layers.

A new cadherin-like protein was identified in oocytes, eggs, and cleavage stage embryos of *Xenopus laevis*. An antiserum raised to a 17 amino acid peptide derived from a highly conserved region in the cytoplasmic domain of all the known cadherins recognized specifically an ~120 kD polypeptide that was distinguishable from E- and N-cadherin by molecular size on SDS gels and by the lack of cross-reactivity of mAbs to E- and N-cadherin. This 120 kD polypeptide has been named "C-cadherin" due to its presence in the cleavage stages of *Xenopus* embryos. A possible relationship to P-cadherin in mouse has not yet been excluded. C-cadherin was present in the large stage 6 oocytes at low level but accumulated in oocytes to the levels found in the egg as a result of progesterone induced maturation. We propose

that C-cadherin may play a role in the Ca^{2+}-dependent adhesion and intercellular junction formation between cleavage stage blastomeres.

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I wish to express my sincere appreciation to my advisor, Professor Barry Gumbiner for his academic advice and encouragement throughout my study at UC San Francisco. His deep inspiration and discussion produced a successful achievement of this study. In addition to his academic support, his warm character and close friendship make our family always happy at San Francisco. I would also like to give many thanks to Professor Judy White and Professor Caroline Damsky for their many helpful advices and comments as committee members. Pierre McCrea, Ted Lowenkopf, and Chunghyun Lee deserve special thanks. They provide not only many technical assistances but also emotional support as colleagues. I greatly appreciate Professor Ray Keller at UC Berkeley for teaching me explantation techniques of *Xenopus* embryos and all the members of Kirschner' lab for providing *Xenopus* egg and microinjection technique. I would like to thank Professor Louis Reichardt at UC San Francisco for providing a peptide of L-CAM which enabled us to study C-cadherin.

My parents gave me a wonderful environment to continue higher education. Finally, I wish to thank my husband Mansoo and my son Kyoungsoo for their sweet love and for giving me a great joy in my life.

TABLE OF CONTENTS

ABBREVIATIONS

ATP	adenosine tri phosphate
BSA	bovine serum albumin
Con A	concanavalin A
DEX	dexamethasone
EDTA	ethylenediamine tetraacetic acid
E-cad100	trypsin resistant ectoplasmic domain of *Xenopus* E-cadherin
FCS	fetal carf serum
GVBD	breakdown of the germinal vesicle
IAA	iodoacetic acid
Ig	immunoglobulin
mAb	monoclonal antibody
kD	kilodalton
MDCK	Mardin-Darby canine kidney
MBSH	modified Barth' solution with Hepes buffer
MBT	midblastula transition
MMR	modified ringer' solution
M phase	mitosis phase
NP-40	Nonidet P-40
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PMSF	phenyl methyl sulfonyl fluoride
SCS	supplemented carf serum
SDS	sodium dodecyl sulfate
S phase	synthesis phase

TCA	trichloroacetic acid
umt	trypsin resistant ectoplasmic domain of canine E-cadherin
ZA	zonula adherens junction
ZO	zonula occludens junction

Chapter 1

The Role of Cadherins in the Assembly of Intercellular Junctions of Epithelia and in the Early Stages of Embryonic Development.

I. Epithelial junctional complex

Epithelial tissues are derived from all three primary germ layers. One of the common characteristics of epithelial cells is that they are interconnected to each other by different types of intercellular junctions; the gap junction, the desmosome, the *Zonula adherens* or intermediate junction, and the *Zonula occludens* or tight junction (Fig. 1). The gap junction and the desmosome are scattered along the entire lateral cellular surface (26). The gap junction is a communicating junction, which mediates transcellular passage of smaller molecules. The desmosomal junctions appear to act like rivets holding the cells together. Electron dense plaques are found both in the intercellular space between the interacting membranes of the desmosomes and on the cytoplasmic side associated with bundles of cytokeratin intermediate filaments (13, 48). The network of cytokeratin filaments and desmosomal junctions may be important for the maintenance of the shape and intergrity of the epithelial sheet. The *Zonula adherens* (ZA) and *Zonula occludens* (ZO) are restricted to the most apical region of the lateral boundary between epithelial cells (26). The ZO presumably is responsible for the transepithelial permeability barrier and the major transepithelial electrical resistance (12). Freeze fracture and electron microscopy of the ZO have shown a series of anastomosing strands present at the cytoplasmic surface of the ZO (75) and two adjacent lateral cellular membranes in very close apposition (26). The ZA is located just below the ZO. A contractile bundle of actin filaments is associated at the cytoplasmic side of the ZA, which extends circumferentially along the cytoplasmic surface of the junctional

membrane (14, 41, 59, 95, 98). Because of their close spatial relationship, the ZO and ZA are functionally related.

Transporting epithelia such as kidney epithelia and intestinal epithelia form permeability barriers between the luminal and serosal spaces by restricting the paracellular diffusion of ions and neutral molecules (12, 21, 24, 56, 58). The permeability barrier is mainly maintained by a tight seal between the luminal and serosal spaces formed by the tight junction. The plasma membrane of the epithelial cells is thus divided into two functionally and biochemically distinct domains; the apical domain facing the lumenal space and the basolateral domain facing the serosal space (21, 33, 84). The surface membrane proteins of the apical and the basolateral domains are distributed in a polar fashion (84). These properties seem important for the normal function of epithelia by preventing back diffusion of active transport of solutes and small molecules, and also by maintaining vectorial transport of those molecules. Two intercellular junctions, the ZO and the ZA are probably responsible for the maintence of permeability barrier and surface polarity. They are spatially associated and completely circumscribe the apex of the cell. Therefore the ZO and ZA have been proposed to function coordinately to seal off and polarize the cells (32, 35).

Association of the cytoskeleton with the ZA seems to be important for the function of the ZA. The ZA associated microfilaments are connected with the plasma membrane probably through an alpha-actinin (14) and vinculin rich plaque (30, 59, 95). Depletion of Ca^{2+} ions from the culture medium of epithelial cells results in rapid

splitting of intercellular junctions and also detachment of the vinculin and actin containing filament bundles from the cytoplasmic faces of the plasma membranes of the ZA (95). The ZA associated actin bundle itself contains myosin, filamin, and tropomyosin, which may be implicated in the ATP- dependent contraction of this microfilament bundle (59), a phenomenon analogous to ZA contraction in corneal epithelium (71). The association of the ZA with the contractile system is also thought to be essential for the morphogenetic contraction of epithelial sheets (1, 100), which may be driven by the sum of the directional contractile forces of individual cells (90). However the function of the ZA is not clear. Because of its close relationship with the ZO and association with the contractile actin filaments, it was proposed by Mooseker that the constriction of the ZA may modulate the assembly and permeability of the ZO (35).

II. The role of E-cadherin in the assembly of intercellular junctions in epithelia

An epithelial cell adhesion molecule E-cadherin was first identified because it mediates the Ca^{2+} dependent process of compaction in the early mouse embryo (17, 44, 69). The compaction process, which occurs at the eight-cell stage, is a prerequisite for the establishment of the trophectodermal and inner cell mass cell lineages (46). The blastomeres of early eight-cell embryos are spherical, rather loosely associated, and can be readily dissociated mechanically. During compaction the outer blastomeres maximize their cell contacts and estabilish junctional complexes including desmosomes and tight junctions (22). After compaction, the outer cells become polarized and

have distinct basolateral and apical membrane domains (46, 94). Thus, after compaction an epithelial cell layer of polarized cells surrounds the unpolarized inner cells of the embryo. The process of compaction is disturbed by anti-uvomorulin (E-cadherin) antibodies (46, 83) and by the removal of Ca^{2+} ions (23). However, the expression of E-cadherin on the cell surface does not seem to be correlated with the onset of compaction (93). E-cadherin is maternally stored in the unfertilized eggs and its zygotic synthesis starts at the two cell-stage. Compaction is accompanied by a redistribution of E-cadherin to the cell-cell contact site on the cell surface of blastomeres and recruitment of E-cadherin to the ZA. Therefore, the localization of E-cadherin at the ZA occurs during the formation of the tight polarized transporting epithelium.

E-cadherin seems to play a major role in the assembly of the epithelial junctional complex. The expression of E-cadherin at later stages of development and in adult tissues is restricted to the epithelial cell lineage, independent of germ layer origin (72, 94, 103). In adult mouse intestinal epithelia, E-cadherin (uvomorulin) is localized predominantly in the ZA junction and to a lesser extent at the basolateral membrane, but it is not found at the apical membrane domain of these cells (6). In contrast, it is diffusely distributed on the basolateral surface of Madin-Darby canine kidney (MDCK) cells (34). The function of the diffuse distribution is not known, but it may help in the assembly of other junctions. Polyclonal antibodies against canine E-cadherin inhibit the formation of the ZO, the ZA and desmosomes of MDCK cells in a Ca^{2+} switch experiment (36). Therefore, E-cadherin as a primary adhesive component of the ZA in

association with a contractile actin microfilament bundle may exert the initial force that brings cells into close enough contact to allow the assembly of the other junctions.

III. The Ca^{2+} dependent cell adhesion molecules, cadherins, constitute a gene family

The Ca^{2+}-dependent cell adhesion molecules called cadherins have been identified in many species and different tissues. Three cadherins (E-, N-, and P-cadherin) have been identified so far. E-cadherin (102), which is probably the same as uvomorulin (44), L-CAM in chicken (27), and CAM 120/80 in human (17), is mainly expressed in epithelial tissues in the early mouse embryo and in chicken embryonic cells. N-cadherin (38, 40), which is probably the same as A-CAM in chicken (97), is expressed in most neural tissues, cardiac muscle, and lens. P-cadherin (67) is expressed in the placenta, mesothelium, epidermis, and transiently in many embryonic tissues. Each subclass is detected in a variety of tissues derived from all three germ layers. Many cell types express multiple cadherins simultaneously and their combination differs with cell type (90). For example, the lens epithelium and the visceral cleft express E- and N-cadherin, the basal germ layer of epidermis and the inner ear primordium express E- and P-cadherin. Therefore cadherins are not strictly tissue specific molecules, but rather their expression seems to be regulated spatiotemporally in relation to morphogenetic events (25).

All cadherins have similar biochemical but distinct immunological properties. Most cadherins are ~120 - 140 kD transmembrane

glycoproteins. The antibodies generated to one type of cadherin in one species often (but not always) cross-react with its homologue in other species (11). Cadherins undergo a Ca^{2+}-dependent change in molecular conformation, which can be assayed by Ca^{2+}-dependent trypsin sensitivity (34, 43) and reactivity with conformation specific monoclonal antibodies (11, 43). The Ca^{2+}-dependent trypsin sensitivity has often been used as a criterion for the identification of Ca^{2+}-dependent cell adhesion molecules. The Ca^{2+}-dependent conformational change of cadherins is believed to underline the Ca^{2+}-dependence of the intercellular adhesion events (35). Even though different types of cadherins share much structural similarity, they were found to have distinct immunological specificities and tissue distributions. Antibody mapping experiments showed that the immunological differences between cadherin subclasses reside in the N-terminal regions of the cadherin polypeptides (90).

Cadherins constitute a gene family. E- (61), N- (37), and P-cadherin (68), and L-CAM (28) have been cloned so far. Extensive homologies between species as well as between subclasses were observed (53). The cytoplasmic portion of all cadherins have the greatest homology. This suggests that this region is important in cadherin function, such as its association with common intracellular cytoskeletal components. It has been proposed by many groups that the cytoplasmic domain of cadherins interacts with a talin-like molecule associated with a vinculin-rich plaque, which in turn binds to an actin microfilament bundle, as is found at the focal contact sites associated with cell to substratum adhesion (90, 98). Some regions of the cytoplasmic domain

are almost identical between subclasses and species (37). A polyclonal serum generated to a peptide derived from one of these regions cross-reacts with most of the known cadherins (L. Reichardt, unpublished observation; B. Gumbiner, unpublished observation). Therefore, other members of cadherins might be expected to be identified using this antibody, because many cells obtained from solid tissues show cadherin-like adhesion activity (90). Another highly conserved property of cadherins is that of Ca^{2+} binding. The E-F hand consensus sequence for a Ca^{2+}-binding site typical of proteins like calmodulin is not present in cadherin sequences. However conserved sequences with negatively charged amino acids in internal repeats may be candidates for the Ca^{2+}-binding sites (37, 78).

Cadherins may bind in a homophilic manner. Transfection of E- and P-cadherins into L-cells, which normally have little cadherin activity, causes cellular aggregation of the resulting transfectants (66). Transfected cells preferentially adhered to cells expressing the same cadherin subclass and not to untransfected L-cells (90). However coculturing L-CAM-containing liver cells with A-CAM (N-cadherin) - containing lens cells allowed the formation of some heterotypic adherens-type junctions (96). In both experiments, the relative affinities of cadherin interactions were not measured and direct biochemical evidence for interactions was absent. Therefore, it is not completely clear whether cadherins interact with both homotypic and heterotypic mechanisms.

IV. *Xenopus* embryos as a model system to study the role of cadherins in early embryonic development

The early stages of embryonic development in the frog *Xenopus laevis* provide a special opportunity to investigate the functional role of E-cadherin in the assembly of intercellular junctions. First, the early amphibian embryo undergoes a series of synchronous reductive cleavages until the midblastula transition at the 4000 cell stage (Fig. 2). The early blastomeres cycle periodically through M and S phases without the intervening G phase. The egg stores many components that it needs to build the cells of the embryo, including new surface membrane, histones, etc. (4, 85, 99, 101). Second, unlike early mammalian embryos, early amphibian embryos do not undergo a compaction step. Rather, the blastocoel cavity begins to form during the first cleavage of the fertilized egg (49, 50, 81, 85). Third, the normal environment for the developing amphibian embryo is very dilute pond water. Close cell contact and the assembly of the intercellular junctions is required for the formation of blastocoel cavity. Fourth, the early blastomeres of *Xenopus* embryos are held together by a Ca^{2+}-dependent adhesion mechanism (62, 64, 65).

The blastula of *Xenopus laevis* acts like a transporting epithelium (Fig. 3). The internally facing surface membrane of the blastomeres arises during extension of the cleavage furrow from the fusion of intracellular vacuoles and vesicles with the cell surface (9, 86, 87). The newly added membrane has physiological properties very distinct from the preexisting cortical membrane of the egg. The cortical membrane of the egg is very impermeable to drugs and small molecules and has very high specific resistance (~ 75 $k\Omega\ cm^2$) (19, 20, 86). In contrast, the new membrane is more like a normal plasma membrane.

Electrophysiological studies have provided clear evidence that occluding junctions form to seal off the nascent blastocoel even before the first cleavage is completed (76, 86). The new membrane can be considered to be equivalent to the basolateral domain of epithelia because it contains both the junctional regions and regions that face the internal milieu of the blastocoel. The preexisting egg membrane can be considered to be equivalent to the apical membrane domain because it remains in contact with the external environment. Therefore, the rapid assembly of new membranes from maternal stores and the early appreance of occluding junctions during the early embryonic development of *Xenopus laevis* will provide a good *in vivo* system for studying the role of cadherins in the process of epithelial cell-cell adhesion and the subsequent assembly of occluding junctions.

Formation and expansion of the blastocoel is important for normal development. Na^+-K^+ ATPase in the new surface membrane transports Na^+ ions from cytoplasmic stores of embryo, which are probably the yolk platelets, into the blastocoel cavity (60). Active transport of Na^+ causes the passive movement of water along the osmotic gradient from the outside medium. As a result of this redistribution of Na^+ ions, the Na^+ and K^+ content of the blastocoel fluid resembles that of adult plasma. Incubating cleaving embryos in hyperosmotic media (~120 mOsm) prevents the blastocoel cavity from expanding, due to the inability of water to follow Na^+ into the blastocoel (60). Microinjection of ouabain or metabolic inhibitors of active transport, such as dinitrophenol or sodium azide, causes the cells to swell and eventually lyse by inhibiting the expansion of the

blastocoel cavity. The few embryos which do not die turn into acephalic tadpoles with swollen heart chambers (87).

Amphibian embryos have also been successful experimental models for the study of cell adhesion and cell behavior in morphogenetic events such as gastrulation (31). In classical embryology, early amphibian embryos were used to demonstrate the importance of selective cell adhesion in morphogenesis (88, 92). More recently *Xenopus* embryos have been used to study the developmental regulation of gene expression and the molecular biology of tissue induction and differentiation (18). However little is known about cell adhesion molecules in *Xenopus laevis* or other amphibians. E-cadherin is the earliest adhesion molecule expressed in mouse and chicken embryos (39, 91, 93). Therefore, it was expected that E-cadherin would be stored in *Xenopus* eggs and involved in cell-cell adhesion of early blastomeres.

Figure 1. A diagram of epithelial junctional complexes (32).

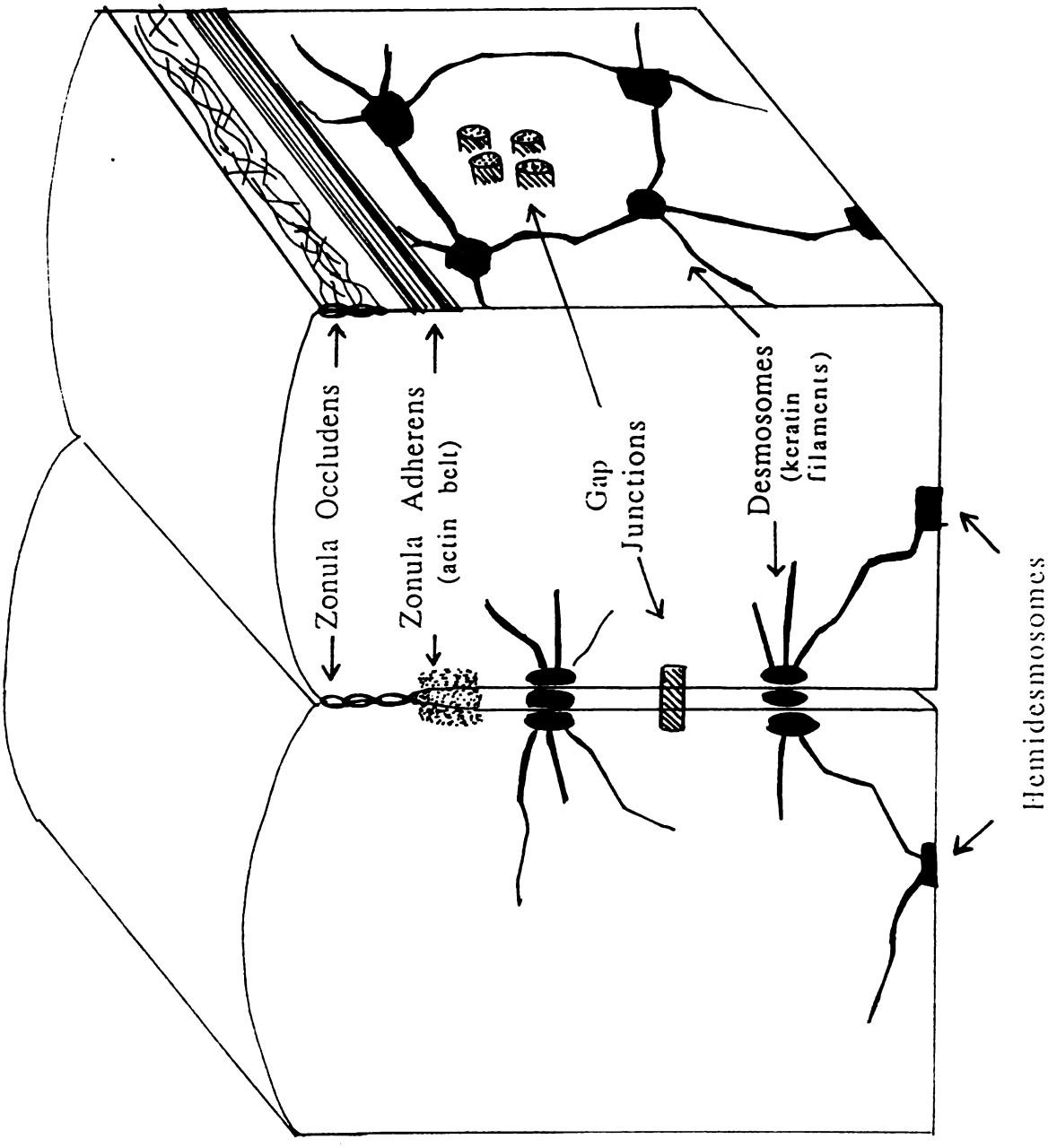

Zonula Occludens
Zonula Adherens
(actin belt)
Gap
Junctions
Desmosomes
(keratin
filaments)
Hemidesmosomes

Figure 2. Major temporal events in the early development of the *Xenopus* egg (53).

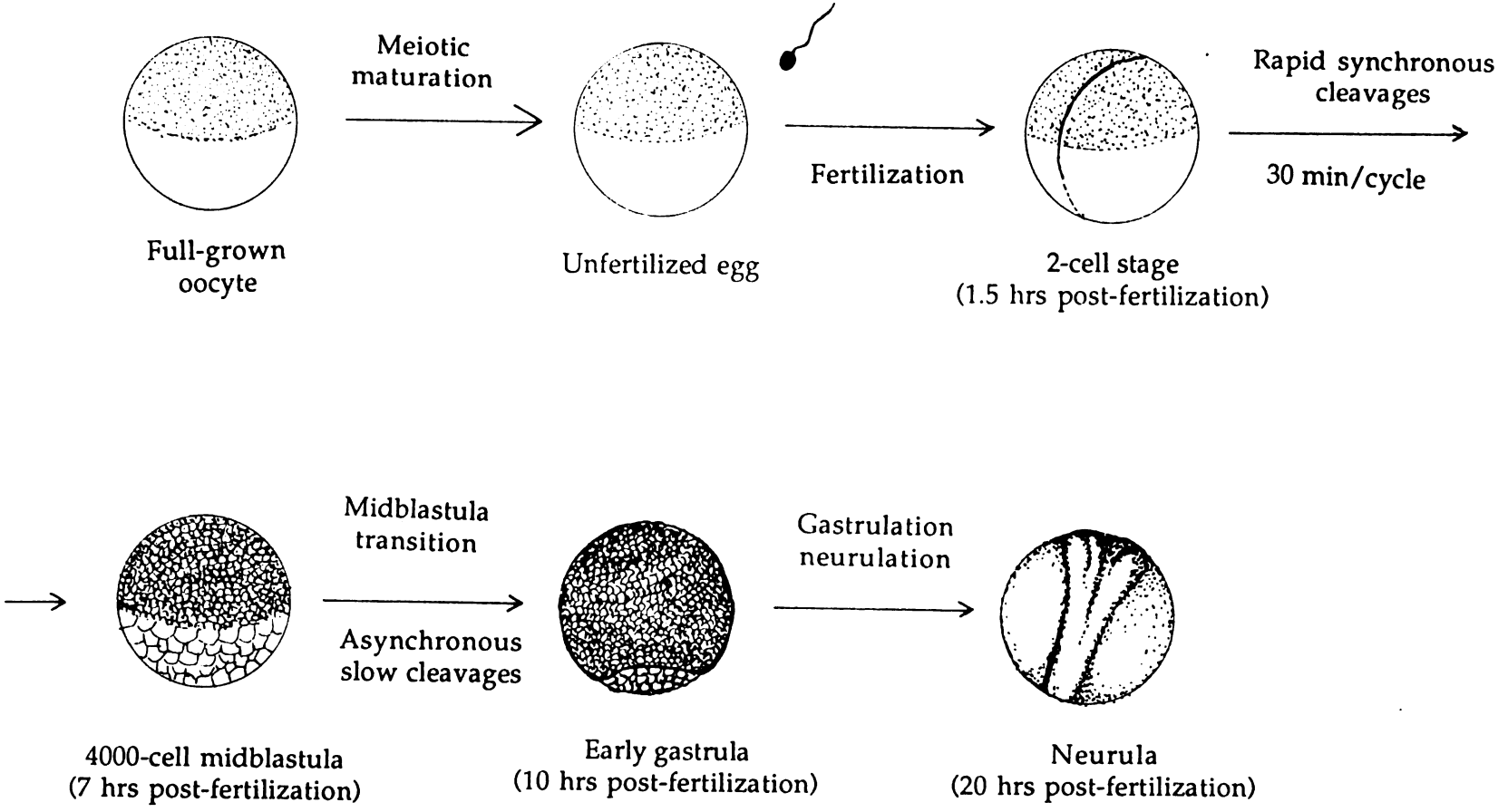
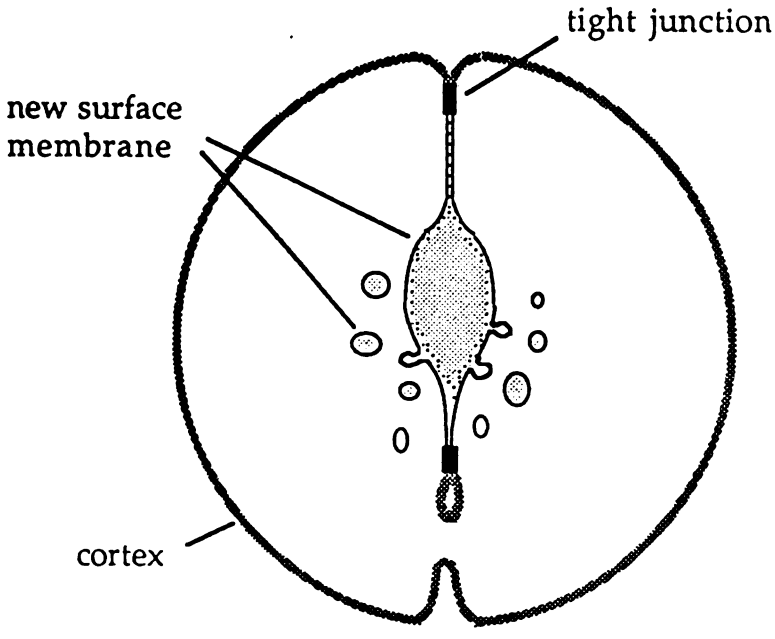

Meiotic maturation
Fertilization
Rapid synchronous cleavages
30 min/cycle
Full-grown oocyte
Unfertilized egg
2-cell stage
(1.5 hrs post-fertilization)
Midblastula transition
Asynchronous slow cleavages
Gastrulation neurulation
4000-cell midblastula
(7 hrs post-fertilization)
Early gastrula
(10 hrs post-fertilization)
Neurula
(20 hrs post-fertilization)

Figure 3. A diagram of cleaving embryo at two cell stage.



egg cleavage furrow

Chapter 2.

Identification and Purification of E-cadherin from the A6 *Xenopus* Kidney Cell Line.

Summary

Xenopus E-cadherin was identified in the A6 *Xenopus* kidney cell line by antibody cross-reactivity and its Ca^{2+}-dependent trypsin sensitivity. A polyclonal serum to canine E-cadherin was used for the identification of *Xenopus* E-cadherin. Since the titer of cross-reacting antibodies was low, specific enrichment of the cellular glycoprotein fraction was necessary to detect *Xenopus* E-cadherin by immunoblotting. The polyclonal serum recognized a 100 kD trypsin resistant fragment (E-cad100) released from the cell surface by trypsin in the presence of Ca^{2+}. The E-cad100 was completely degraded by trypsin in the absence of Ca^{2+}. E-cad100 was the only major Con A binding glycoproteins released from the cell surface by tryptic digestion in a Ca^{2+}-dependent manner. The intact E-cadherin molecule was a 140 kD glycoprotein, which preferentially distributed into the aqueous phase of Triton X-114 extracts of A6 cells, like E-cadherin in other species. Three polypeptides (140 kD, 116 kD, 100 kD) in the aqueous phase recognized by the polyclonal serum contained the E-cadherin epitopes. The lower molecular weight polypeptides (116 kD &100 kD) are probably degradation products of the 140 kD intact E-cadherin. The polyclonal serum to canine E-cadherin was good enough for the identification of *Xenopus* E-cadherin in A6 cells but not adequate to study the role of E-cadherin in the early *Xenopus* embryo. In order to generate mAbs specific to *Xenopus* E-cadherin, E-cad100 was purified from A6 cells. Dexamethasone, which induced high transepithelial electrical

resistance of A6 monolayers, did not change the steady state levels of the E-cadherin polypeptide. Therefore A6 cells cultured in normal growth medium were used for purification of E-cad100. The supernatant of tryptic digests of whole cells was fractionated on a DE-52 column, concentrated by binding to Con A-sepharose-4B, and separated by SDS-PAGE. ~ 3μg of E-cad100 was obtained from $2x10^8$ cells.

Introduction

E-cadherin is a cell adhesion molecule that has been implicated in several Ca^{2+}-dependent cell-cell interactions between epithelial cells. E-cadherin belongs to a family of Ca^{2+}-dependent cell adhesion molecules known as the cadherins, which includes N-cadherin in neural tissues (38), P-cadherin in placenta (67), and probably others yet to be identified. These cadherins are highly related at the amino acid sequence level and probably perform similar functions in different tissues (90). They seem to be particularly important for cell interactions that occur during embryological development, because changes in the expression of cadherin type are often associated with morphogenetic events in embryogenesis (39, 67, 91). The cadherins have been proposed to be responsible for the specific adhesion and intercellular recognition among like cells that regulates the formation and segregation of tissues during morphogenesis (90).

Early developing embryos of the amphibian *Xenopus laevis* provide particularly good experimental material for investigating the behavior of epithelial cells and the role of intercellular adhesion during development. Surprisingly little is known about cell adhesion molecules in *Xenopus laevis* or other amphibians. I undertook in this study to identify *Xenopus* E-cadherin in the A6 *Xenopus* kidney epithelial cell line. Until this investigation, cadherins had not yet been identified in the amphibian system. Cadherins are well conserved between species. Therefore a certain fraction of antibodies raised

against E-cadherin in one species may cross react with its homologue in another species. One of the common biochemical properties of cadherins is their Ca^{2+}-dependent conformational change, which can be assayed by the Ca^{2+}-dependent trypsin sensitivity (34, 43). Therefore, this Ca^{2+}-dependent conformational change and the cross-reactivity of antibodies were employed as criteria for the identification of E-cadherin in *Xenopus laevis*.

Results

I. Identification of E-cadherin in the A6 *Xenopus* kidney cell line.

A polyclonal serum generated against canine E-cadherin (34, 36) was used to identify E-cadherin in the A6 *Xenopus* kidney cell line. Titers of cross reacting antibodies were too low to detect *Xenopus* E-cadherin by immunoblotting of the total SDS extracts of A6 cells and the supernatant of tryptic digests of A6 cells. It was necessary to enrich glycoprotein fractions by binding to Con A-sepharose-4B to identify *Xenopus* E-cadherin in A6 cells (Fig. 1 and Fig. 2).

E-cadherin in murine and canine species can be released from the cell surface as a protease resistant fragment by trypsin in the presence of Ca^{2+} (17, 34, 43). In the absence of Ca^{2+}, it is degraded extensively by trypsin. This Ca^{2+}-dependent change in trypsin sensitivity was also used as a criterion for identification of E-cadherin in *Xenopus laevis*. Whole A6 cells were digested with trypsin in the presence or absence of Ca^{2+}, and proteins released from the cell surface were concentrated with Con A-sepharose-4B. A 100 kD polypeptide was recognized by polyclonal antibodies against canine E-cadherin (Fig. 1, lane a), but not detected in the supernatant of cells trypsinized in the absence of Ca^{2+} (lane b). Like E-cadherin in other species (17), *Xenopus* E-cadherin bound to the lectin Con A, but did not bind to the lectin wheat germ agglutinin (lane c-e).

The polyclonal serum to canine E-cadherin had a much weaker ability to precipitate the 100 kD trypsin resistant fragment of *Xenopus* E-cadherin than the lectin Con A (compare lanes a and c in Fig. 2). ^{35}S-methionine labelled A6 cells were trypsinized in the presence or in the absence of Ca^{2+}, and the supernatants were either bound to Con A-sepharose-4B or immunoprecipitated with polyclonal serum to canine E-cadherin or preimmune serum. Trypsin can cleave many glycoproteins from the cell surface even in the absence of Ca^{2+} (lane b). E-cadherin was the only major glycoprotein released from the A6 cell surface that possesed the Ca^{2+} dependent change in trypsin sensitivity (lane a).

To identify the intact E-cadherin protein, Triton X-114 extracts of A6 cells were phase-partitioned and the glycoproteins in the aqueous phase were concentrated by binding to Con A-sepharose-4B. Similar to mouse and canine E-cadherin (34, 72), *Xenopus* E-cadherin was preferentially distributed in the aqueous phase of Triton X-114 extracts (Fig. 7, lane d-f) even though it is known to be an integral membrane protein (37, 78). Two polypeptides (140 kD, 116 kD) were recognized by polyclonal serum to canine E-cadherin, and two other polypeptides (100 kD, 80 kD) were recognized weakly (Fig. 3, lane a). None of these polypeptides was recognized by the preimmune serum (lane c). After microaffinity purification on highly purified trypsin resistant ectoplasmic domain of canine E-cadherin (umt) (36) by the Olmstead procedure (8), anti E-cadherin polyclonal antibodies recognized three polypeptides (140 kD, 116 kD, 100 kD) (lane b). Therefore, these three polypeptides contain E-cadherin epitopes.

The 140 kD polypeptide in the aqueous phase of Triton X-114 extracts of A6 cells was confirmed to be the intact parent molecule to the 100 kD trypsin resistant fragment released from cell surface by trypsin in the presence of Ca^{2+}(Fig. 4). The aqueous phase of Triton X-114 extracts of A6 cells was digested with trypsin in the presence or absence of Ca^{2+}. All three polypeptides in the aqueous phase of Triton X-114 extracts were completely degraded by trypsin in the absence of Ca^{2+} (lane d and e). In the presence of Ca^{2+}, two trypsin resistant fragments (107 kD, 100 kD) were produced (lane c). The molecular mass of the major trypsin resistant fragment from digestion in solution is higher than that from digestion of the whole cell (compare lane a and c). It was not determined whether the trypsin resistant fragment of higher molecular mass was generated in solution because an additional tryptic site otherwise buried in the plasma membrane is exposed when E-cadherin is solubilized.

Three polypeptides (140 kD, 116 kD, 100 kD) in the aqueous phase exhibited the Ca^{2+}-dependent sensitivity to trypsin digestion. The 116 kD and 100 kD polypeptides are probably degradation products of the 140 kD polypeptide because extraction of cells with boiling SDS buffer greatly reduced the levels of these bands (Fig. 3 in Chapter 3). These criteria, therefore, identify the 140 kD polypeptide as *Xenopus* E-cadherin and the 100 kD polypeptide as its trypsin resistant ectoplasmic domain (E-cad100). The molecular masses of both polypeptides are ~ 20 kD higher than those in other species (3, 17, 27, 34, 70, 72).

II. Effect of dexamethasone on the expression of E-cadherin in A6 cells.

Dexamethasone promotes "tight" monolayers of A6 cells. The tightness of the monolayer may be monitored by measuring its transepithelial electrical resistance (34). When A6 cell monolayers were cultured on filters in normal A6 medium supplemented with 10^{-7} M dexamethasone, the transepithelial electrical resisitance was much higher than that of A6 monolayers cultured in normal medium (Fig. 5). At seven days of culture, it was ~ 20 times higher than in the normal medium. The high transepithelial electrical resistance of A6 cells dropped down to the levels of a bare filter after a 10 min incubation in Ca^{2+}-free medium. The cell densities in both media were indistinguishable based on counting of nuclei stained with Hoechst dye. The morphology of A6 cells cultured in medium supplemented with dexamethasone was slightly different from their morphology in normal medium (Fig. 6). A6 cells grown in the presence of dexamethasone had darker boundaries when observed by phase microscopy.

It has been proposed that E-cadherin regulates the assembly of intercellular junctions in MDCK cells (36). To determine whether dexamethasone induces higher levels of E-cadherin expression (which would also aid in its purification), the levels of E-cadherin expression in its presence and absence were examined. Based on the immunoblotting assay of Triton X-114 extracts of A6 cells (Fig. 7), the level of E-cadherin expression was similar in the cells cultured with or without dexamethasone. Therefore, induction of high transepithelial electrical resistance by dexamethasone treatment may be due to changes in the expression of other components such as tight junction proteins.

III. Purification of E-cad100 for the generation of monoclonal antibodies

The polyclonal serum to canine E-cadherin was adequate for the identification of *Xenopus* E-cadherin but it was necessary to generate mAbs specific to *Xenopus* E-cadherin for further study of E-cadherin in the early *Xenopus* embryo. For the generation of mAbs, E-cad100 was purified from the supernatant of trypsin digests of A6 cells in the presence of Ca^{2+}. E-cad100 was eluted from a DE-52 column with 0.1 M - 0.2 M NaCl (Fig. 8), concentrated by binding to Con A-sepharose-4B, and then purified by SDS-PAGE. In one preparation approximately 3 μg of E-cad100 was obtained from 2×10^8 cells (Fig. 9, lane a). Immunoblotting of the same gel with the polyclonal antiserum against canine E-cadherin showed that the purified polypeptide was the trypsin resistant fragment of E-cadherin (lane b).

Discussion

I wished to undertake the study of E-cadherin (uvomorulin, L-CAM), the Ca^{2+}-dependent epithelial adhesion molecule, in early developing embryos of *Xenopus laevis*. This protein had been identified previously in several other species including mouse (44, 72), chicken (27), human (17), and canine (3, 34). In order to investigate the function of E-cadherin in developing *Xenopus* embryos, it was first crucial to unambiguously identify the same protein in this species. The identification of E-cadherin in *Xenopus laevis* in this study was established by several criteria. First, antibodies raised to canine E-cadherin recognized the homologous protein in the *Xenopus* A6 kidney epithelial cell line. Second, this protein exhibited the Ca^{2+}-dependent resistance to trypsin digestion that is characteristic for the cadherins in all species (34, 43). Third, like E-cadherin in other species (17) it bound to the lectin ConA but not to the lectin wheat germ agglutinin. Fourth, it was preferentially distributed in the aqueous phase of Triton X-114 extracts like mouse and canine E-cadherin (34, 72) even though it is known to be a transmembrane protein (37).

Figure 1. Identification of the 100 kD trypsin-resistant ectoplasmic domain of *Xenopus* E-cadherin (E-cad100). $5x10^6$ A6 cells were digested with trypsin in the presence of Ca^{2+} (lane a, c-e), or in the absence of Ca^{2+} (1.5 mM EDTA) (lane b). The supernatants were bound to Con A sepharose-4B (lane a, b, e) or wheat germ agglutinin sepharose-4B (lane c). The glycoproteins remained in the wheat germ agglutinin unbound fraction was concentrated by binding to Con A sepharose-4B (lane d). The samples were electrophoresed and immunoblotted with a 300 fold dilution of a polyclonal antiserum against canine E-cadherin. Numbers at left are molecular weight markers in kilodaltons. Arrow head = E-cad100

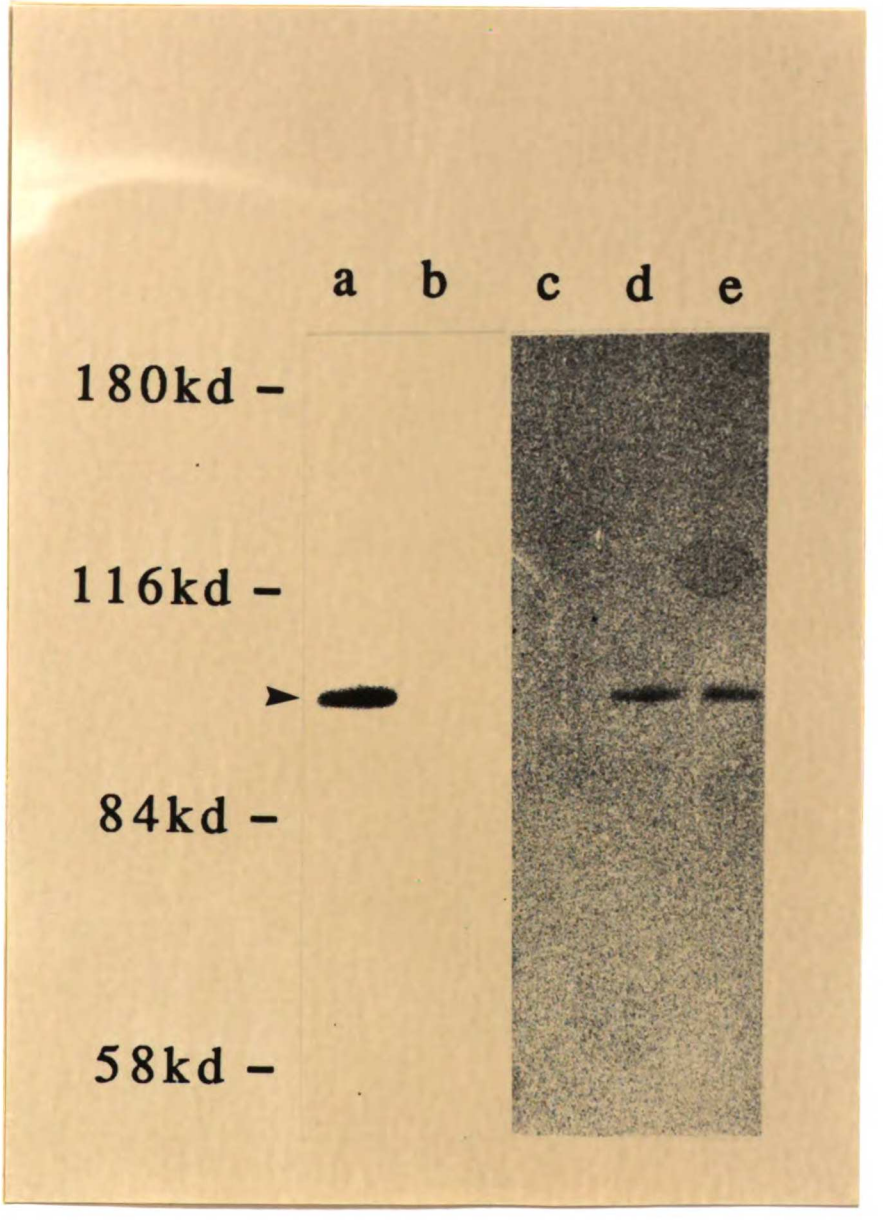

a
b
c
d
e
180kd –
116kd –
84kd –
58kd –

Figure 2. E-cad100 was one of the major Con A binding proteins released from the A6 cell surface by trypsin in the presence of Ca^{2+}. A6 cells were labelled overnight with 0.3 mCi of ^{35}S-methionine and trypsinized in the presence of Ca^{2+} (lane a, c, e) or in the absence of Ca^{2+} (lane b, d, f). The supernatants were bound to Con A sepharose-4B (lane a, b) or immunoprecipitated with the polyclonal antiserum to canine E-cadherin (lane c, d) or with a preimmune serum (lane e, f). Numbers at left are molecular weight markers in kilodaltons. Arrow head = E-cad100.

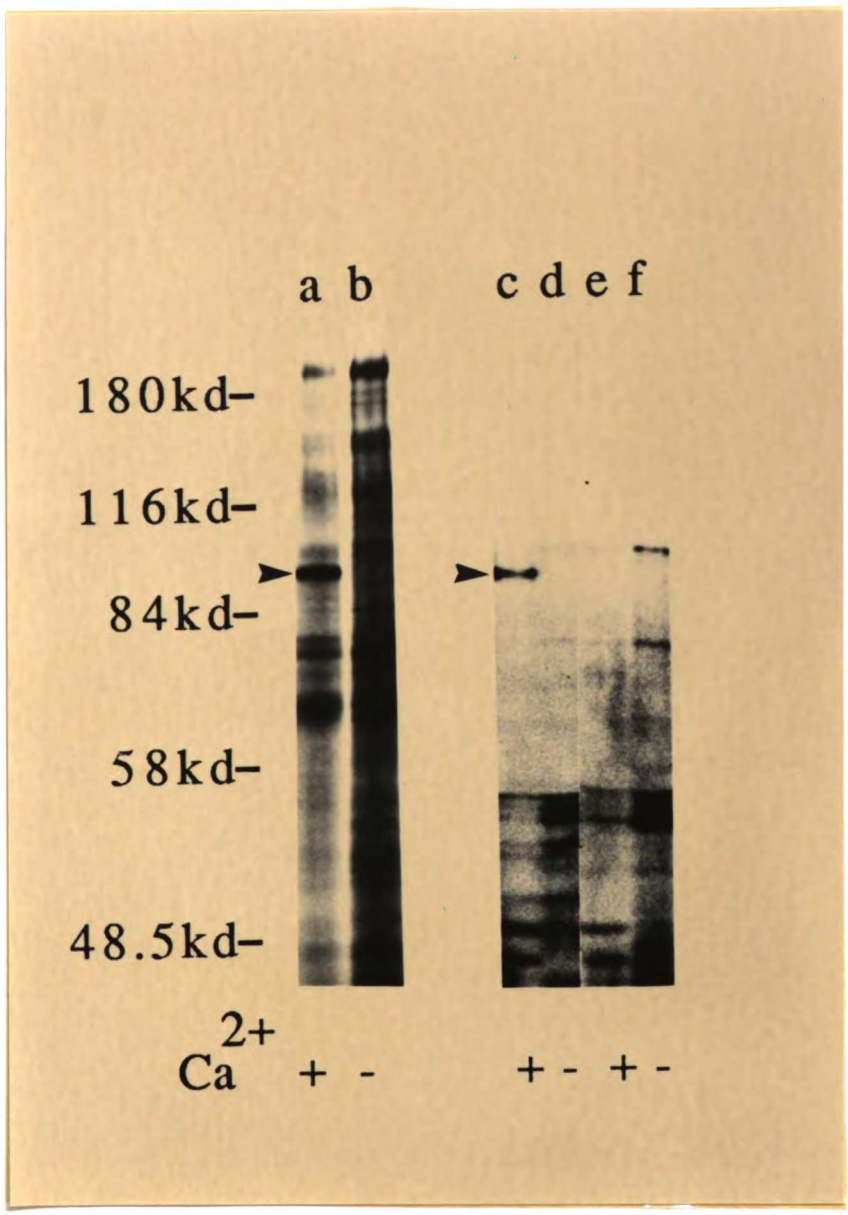

a b
c d e f
180kd–
116kd–
84kd–
58kd–
48.5kd–
2+
Ca
+ -
+ - + -

Figure 3. Identification of the 140 kD intact E-cadherin. Con A bound fractions of the aqueous phase of Triton X-114 extracts of A6 cells were immunoblotted with the polyclonal antiserum to canine E-cadherin (lane a), with the same antibodies after microaffinity purification on canine E-cadherin (lane b), with the preimmunine serum (lane c). Numbers at left are molecular weight markers in kilodaltons.

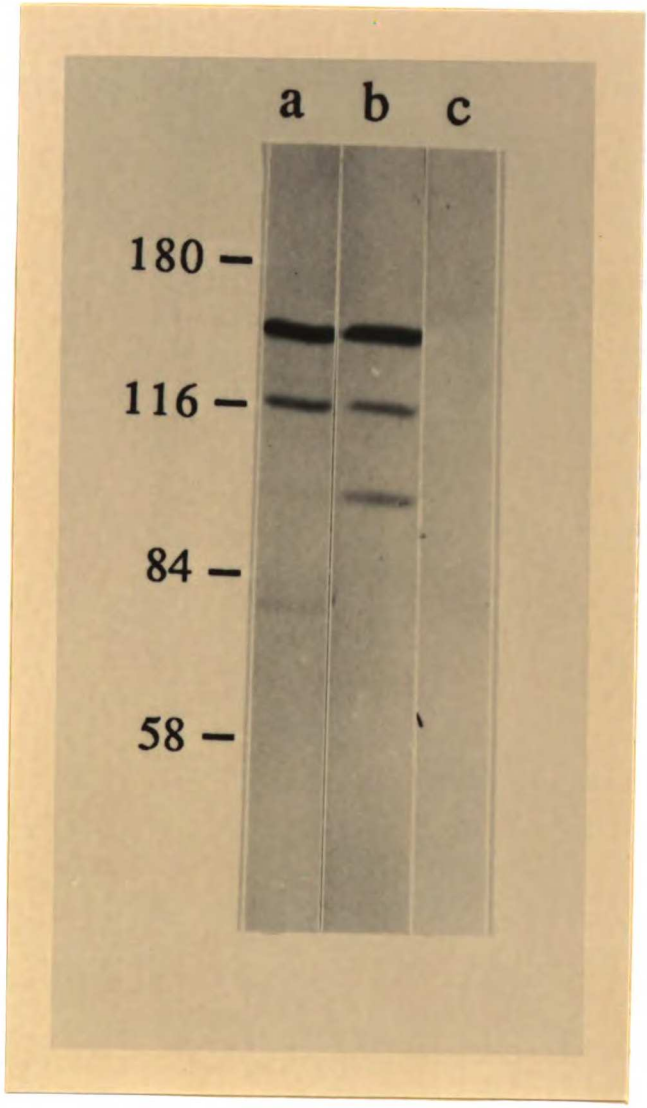

a
b
c
180 –
116 –
84 –
58 –

Figure 4. Ca^{2+} dependent trypsin sensitivity of solubilized E-cadherin. A6 cells were extracted with Triton X-114 containing buffer in the absence of protease inhibitors. The aqueous phase was trypsinized in the presence of Ca^{2+} (lane c) or in the absence of Ca^{2+} (lane d) and the Con A bound fractions were immunoblotted with the polyclonal antiserum. The aqueous phase before trypsinization (lane e) and E-cad100 in the supernatant from whole cell tryptic digestions (lane a and b) are shown for comparison. Numbers at left are molecular weight markers in kilodaltons.

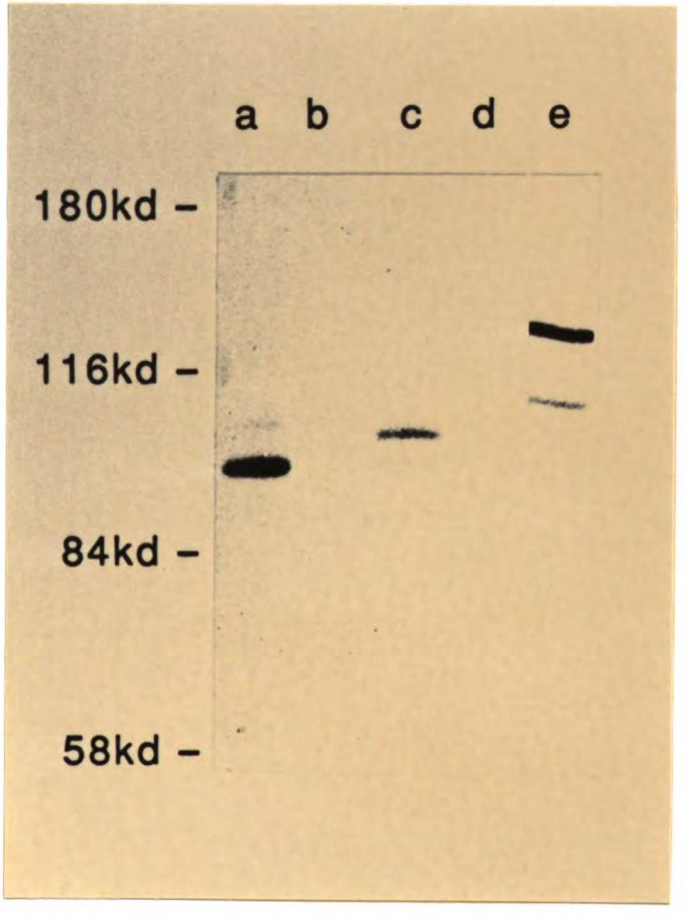

a
b
c
d
e
180kd –
116kd –
84kd –
58kd –

Figure 5. Effects of dexamethasone on the transepithelial electrical resistance of the A6 cell monolayer. A6 cells were cultured on the filters in the presence or in the absence of 10^{-7} M dexamethasone. The transepithelial electrical resistance across the monolayers was measured at indicated day of cell culture. Values are mean, n=2

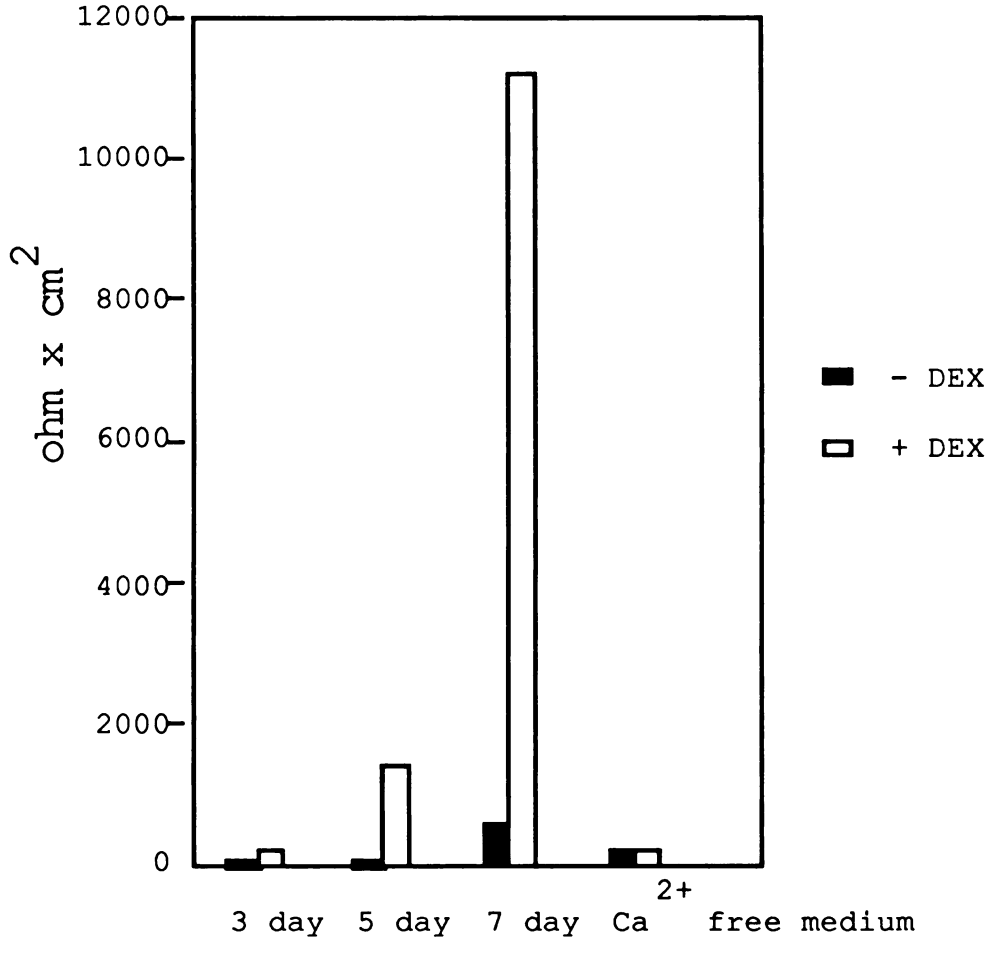

12000
10000
8000
6000
4000
2000
0
ohm x cm 2
- DEX
+ DEX
3 day
5 day
7 day
Ca 2+ free medium

Figure 6. Effect of dexamethasone on the morphology of A6 cells. A6 cells were grown on the cover glasses for 7 days in the presence or in the absence of dexamethasone and examined under the phase contrast microscope.

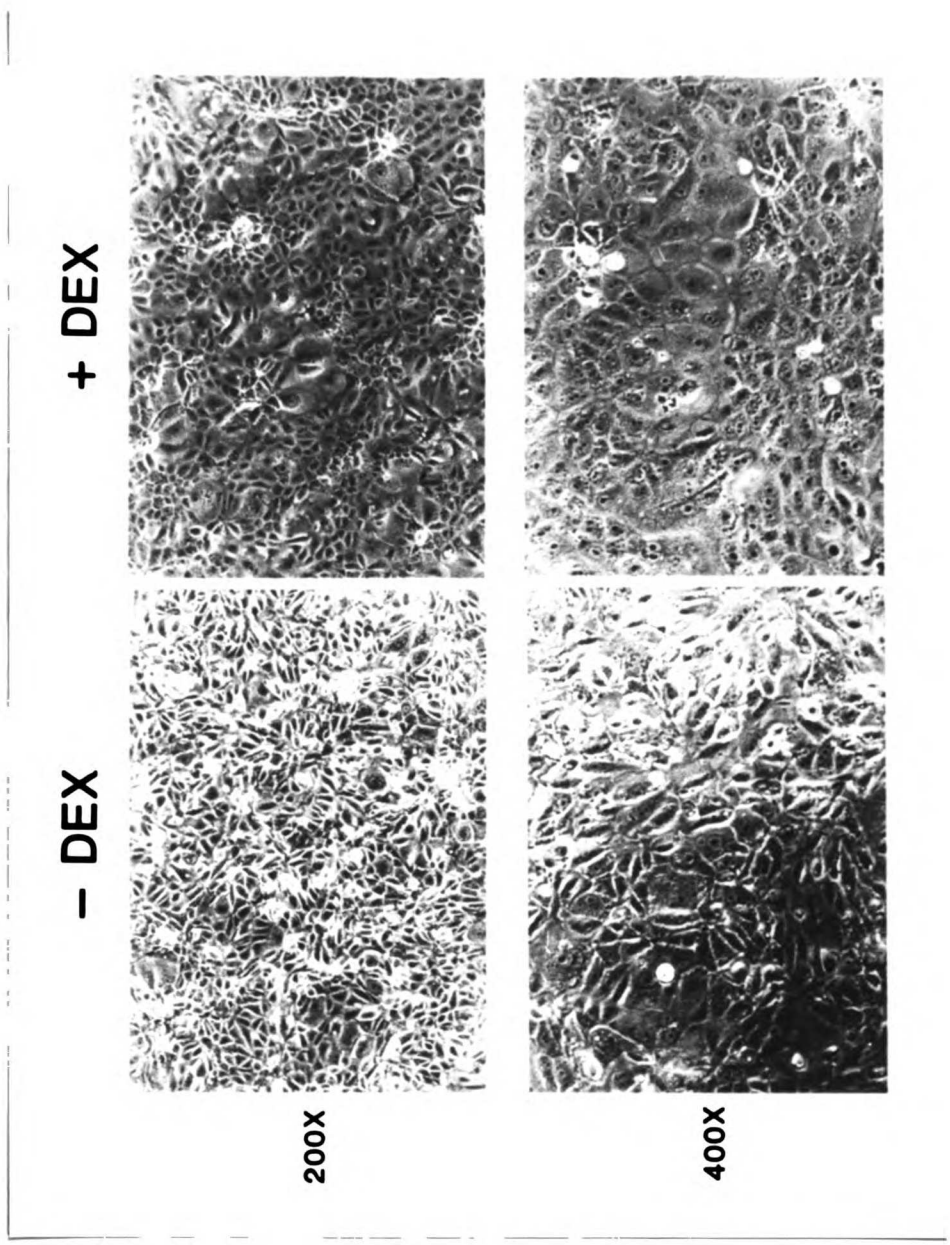

+ DEX
- DEX
200X
400X

Figure 7. Effect of dexamethasone on the levels of E-cadherin expression in the A6 cells. A6 cells grown in the presence (lane a-c) or in the absence (lane d-f) of dexamethasone were extracted with Triton X-114 containing buffer. The aqueous phase were bound to Con A sepharose-4B. The equivalent fractions of the aqueous phase (lane a, d), the insoluble pellet (lane b, e), and the detergent phase (lane c, f) were immunoblotted with the polyclonal serum. Numbers at left are molecular weight markers in kilodaltons.

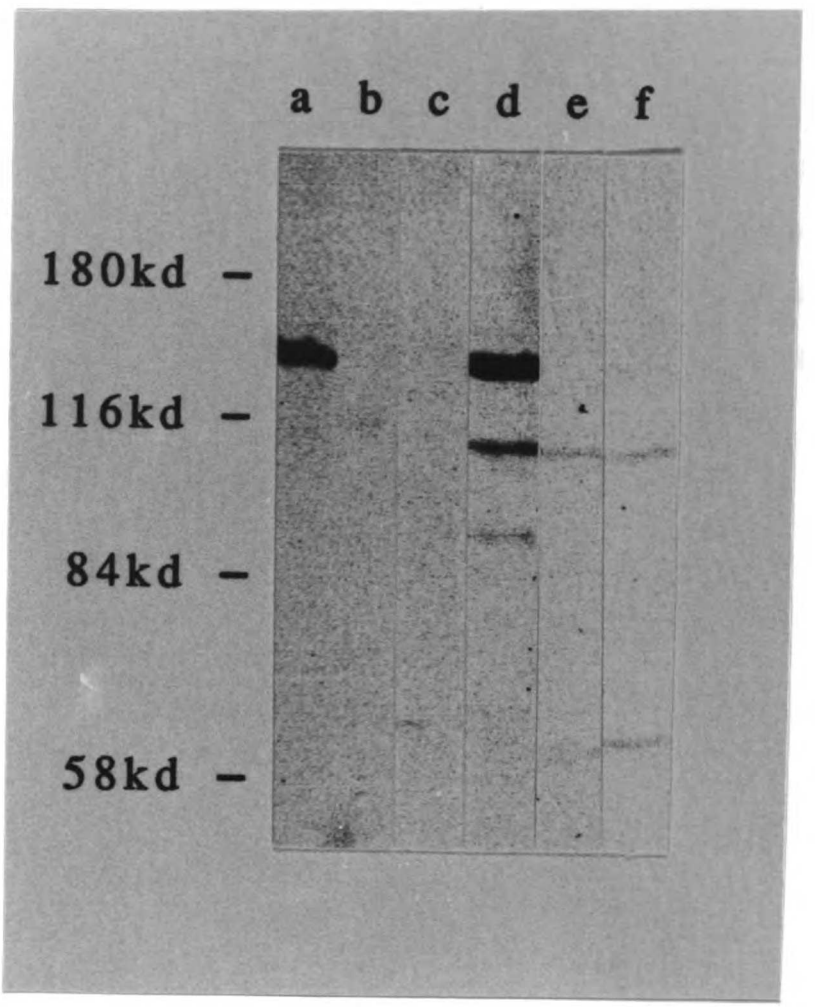

a b c d e f
180kd –
116kd –
84kd –
58kd –

Figure 8. Fractionation of E-cad100 on a DE-52 column. The supernatant of trypsin digests of 2×10^8 A6 cells was equilibrated with the column buffer. The bound fractions were eluted by a linear gradient of NaCl (0.05-0.5 M). 5 µl of each fraction was immunoblotted with the antiserum to canine E-cadherin. Numbers at left are molecular weight markers in kilodaltons.

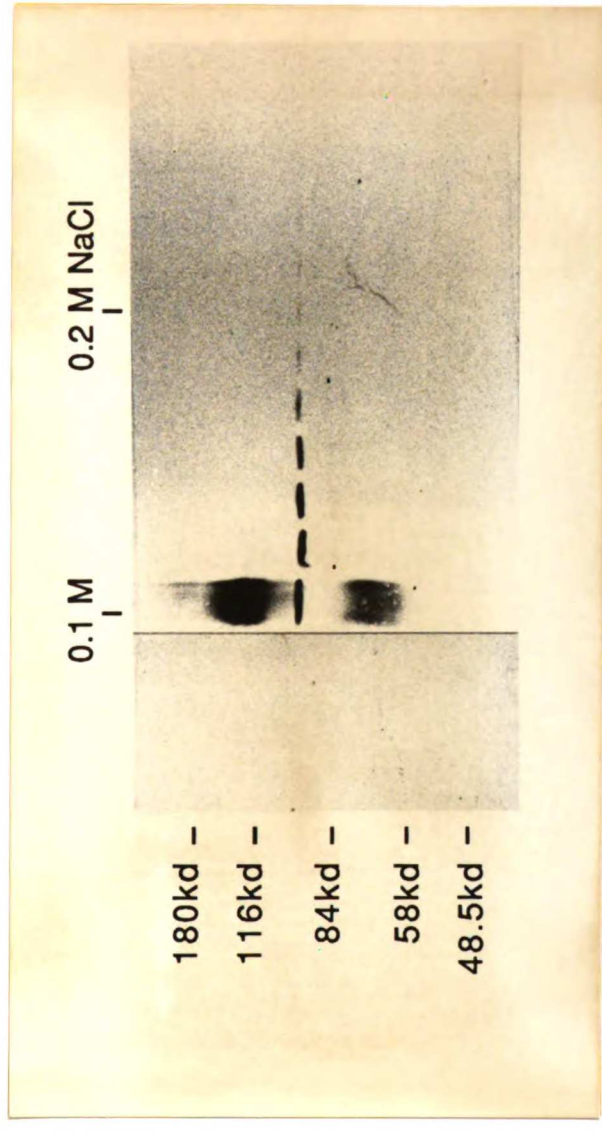
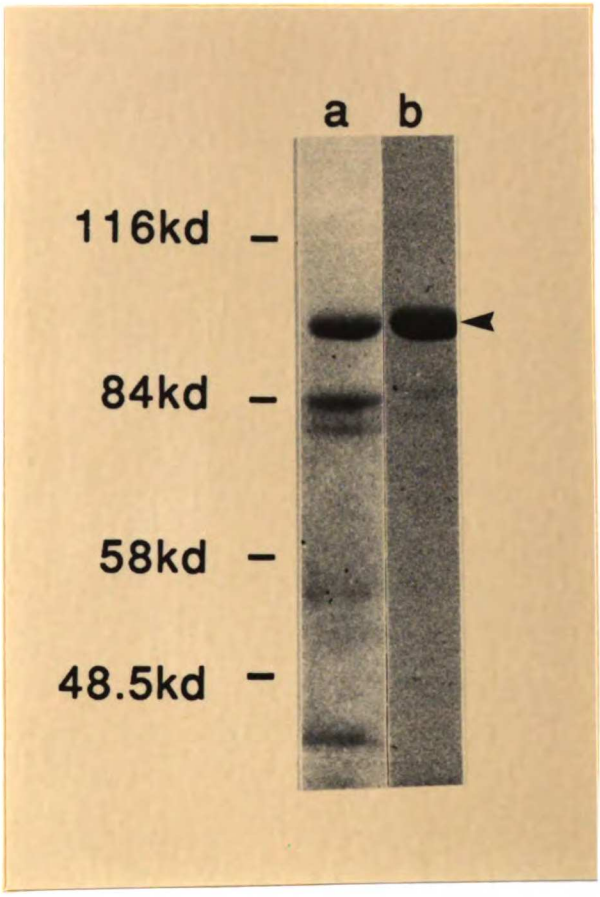

0.1 M
0.2 M NaCl
180kd
116kd
84kd
58kd
48.5kd

Figure 9. Purification of E-cad100. Lane a) Coomassie blue stained gel of one preparation of E-cad100 obtained from $2x10^8$ A6 cells. Lane b) Immunoblotting of the same gel with polyclonal antiserum to canine E-cadherin. Numbers at left are molecular weight markers in kilodaltons.


a
b
116kd
84kd
58kd
48.5kd

Chapter 3

Generation of Monoclonal Antibodies to *Xenopus* E-cadherin and Characterization of *Xenopus* E-cadherin in A6 Cells.

Summary

Five independent monoclonal antibodies specific to *Xenopus* E-cadherin were generated and used for the characterization of *Xenopus* E-cadherin. Two monoclonal antibodies 5D3 and 19A2 recognized both the (+) Ca^{2+} conformation and the (-) Ca^{2+} conformation of E-cad100 in solution. Monoclonal antibodies 8C2 and 31D2 recognized only the (-) Ca^{2+} conformation of E-cad100. This conformational change was reversible, depending on the Ca^{2+} concentration.

Monoclonal antibodies were used to further characterize *Xenopus* E-cadherin. E-cadherin was initially synthesized as a 155 kD presursor and converted into a 140 kD mature form. To assess the effects of the monoclonal antibodies on the adhesive function of E-cadherin between epithelial cells, a resistance recovery assay was employed. The individual monoclonal antibodies or a mixture of mAbs strongly inhibited the recovery of the transepithelial electrical resistance of A6 cell monolayers. E-cadherin was expressed at high levels in other epithelial tissues, but not in brain. Based on indirect immunofluorescence microscopy, E-cadherin was localized at the basolateral plasma membrane of A6 cells, hepatocytes, and alveolar epithelia. Therefore, the monoclonal antibodies I have generated are specific probes for the *Xenopus* epithelial cell adhesion molecule E-cadherin.

Introduction

E-cadherin has been identified in many species (17, 44, 69). In early mouse embryos, it is initially expressed in most cells but its expression becomes restricted to epithelial tissues of later stage embryos and the adult organism (72, 94, 103). During embryonic morphogenesis, the expression of cadherins is regulated temporally and spatially. Many adult tissues express different types of cadherins simultaneously. Therefore, to study the role of E-cadherin in the early *Xenopus* embryos, it is crucial to characterize *Xenopus* E-cadherin with monoclonal antibodies. E-cadherin seems to function at an early step in intercellular adhesion and to be required for the assembly of the entire epithelial junctional complex (36), although it is highly enriched at the *zonula adhaerens* junction in the mouse intestinal epithelia (6). The ability of monoclonal antibodies to exert an inhibitory effect in a resistance recovery assay was used to functionally identify E-cadherin in MDCK cells (34). Therefore, a test of the ability of the monoclonal antibodies to interfere with the reassembly of intercellular junctions provides a functional criterion for the Ca^{2+}-dependent epithelial adhesion molecules. In this study, E-cad100 was purified from the A6 kidney epithelial cell line and used to generate monoclonal antibodies. The mAbs were shown to block the recovery of transepithelial electrical resistance, thereby confirming a role for *Xenopus* E-cadherin in the establishment of intercellular junctions. The distribution of E-cadherin in various epithelial tissues of *Xenopus laevis* and its

basolateral plasma membrane localization were also used as criteria for being an epithelial cell adhesion molecule.

Results

I. Generation of mAbs to *Xenopus* E-cadherin

Balb/C mice were immunized twice with ~ 5 μg of purified E-cad100 and titers were checked by immunoblotting on samples of purified E-cadherin with the mouse serum obtained from tail bleeds (Fig. 1). The sera from two mice showed a strong band at a 100 fold dilution of the serum and a rather weak band at a 1,000 fold dilution. In the first fusion, no feeder cells were plated for the growth of hybridomas and only two of 400 wells showed clonal growth. However in the second fusion, ~ 1 X 10^4 macrophages/well were plated as feeder cells and ~ 200 of 400 wells showed clonal growth. Supernatants were screened by immunoblotting on samples obtained from tryptic digestion of A6 cells. ~ 50 wells were positive. Attempts were made to subclone ten clones which showed the strongest response by limiting dilution. The five positive clones (5D3, 8C2, 15B2, 19A2 and 31D2) grown as a result of plating 0.3 cell/well were chosen as single clones. MAbs 8C2 and 5D3 were subclass IgG1, while mAbs 19A2 and 31D2 were IgG2b.

II. Characterization of *Xenopus* E-cadherin with mAbs.

MAbs were characterized by immunoblotting a total protein SDS extract of A6 cells (Fig. 2). The 140 kD polypeptide was the major band recognized by mAbs 5D3, 8C2, 15B2, 19A2, and 31D2 (Fig. 2, lane a-e). The lower molecular weight minor bands observed before with the polyclonal antiserum were also detected. They probably represent

degradation products because they were more abundant in Triton X-114 extracts of A6 cells, which are exposed to 37°C for the phase separation, than in the boiled SDS extracts (compare Fig 2. with lane a and b in Fig. 3 B). Another minor band migrating at 155 kD, which had not been detected with the polyclonal antibodies, was also recognized. In this particular experiment, mAbs 31D2 and 15B2 appeared to recognize only the 140 kD polypeptide. However, they detected all of the E-cadherin polypeptides in the aqueous phase of Triton X-114 extracts of A6 cells which tend to have more degradation products (not shown).

The E-cadherin polypeptides were well solubilized by 1 % Triton X-114. Very little E-cadherin was found in the Triton X-114 insoluble pellet (Fig. 3 A, lane b). As shown previously (Fig. 7 in chapter 2), the 140 kD E-cadherin partitions preferentially into the aqueous phase of Triton X-114 (lane c). In contrast, the 116 kD polypeptide was preferentially distributed in the detergent phase of Triton X-114 extracts of A6 cells (lane a). The preferential distribution of the 116 kD polypeptide into the detergent phase could be due either to the proteolytic cleavage of a hydrophilic portion of intact E-cadherin or to a loss of associated hydrophilic proteins upon proteolytic cleavage.

III. Identification of the 155 kD polypeptide as the E-cadherin precursor.

The 155 kD polypeptide recognized by anti-E-cadherin mAbs in Figure 2 is a good candidate for a precursor to *Xenopus* E-cadherin, since an E-cadherin precursor has been observed in biosynthetic labelling of mouse (73), chicken (29), human (M. Wheelock, unpublished observation) and canine cells (Chernov-Rogen and Gumbiner,

unpublished observation). To determine whether the 155 kD polypeptide is a precursor of the 140 kD mature E-cadherin, a pulse-chase experiment was carried out (Fig. 4). The 155 kD polypeptide was heavily labelled with ^{35}S-methionine after the pulse, and was progressively chased into the 140 kD mature form. The half life of the precursor was ~ 45 min, and labelling of the precursor was persistent up to 120 min of chase. Processing of the precursor in *Xenopus laevis* was slower than conversion in other species. This slow conversion probably explains the detection of the precursor by immunoblotting. The prominent ~ 100 kD polypeptide in Figure 5, observed only by immunoprecipitation, is distinct from E-cad100, and is a candidate for a cytoplasmic protein that binds to E-cadherin (P. McCrea and B. Gumbiner, unpublished observation). A similar molecular mass polypeptide has also been found associated with E-cadherin immunoprecipitated from cells of other species (73, 94), (B. Gumbiner unpublished observation).

IV. Conformation specificity of mAbs.

MAbs 5D3 and 19A2 could immunoprecipitate E-cad100 from the supernatant of trypsin digests of ^{35}S-methionine labelled A6 cells in the presence of Ca^{2+}. However mAb 8C2 which showed high titer in the immunoblotting assay could not immunoprecipitate E-cad100 in the presence of Ca^{2+} (Fig. 5).

MAbs 8C2 and 31D2 are capable of distinguishing between the Ca^{2+}-dependent molecular conformations of E-cad100 (Fig. 6). It is possible to reversibly induce the (-) Ca^{2+} conformation of E-cad100 following its

isolation in the presence of Ca^{2+}. ^{35}S-methionine labelled E-cad100 was converted into its (-) Ca^{2+} conformation by removing Ca^{2+} with EDTA for 5 min on ice. MAbs 5D3 and 19A2 were able to immunoprecipitate E-cad100 in both the (+) Ca^{2+} and the (-) Ca^{2+} conformations and mAb 15B2 failed in both cases. However, mAbs 8C2 and 31D2 recognized only the (-) Ca^{2+} conformation of E-cad100. After immunoprecipitation, all of the unbound E-cad100 polypeptide could be recovered from the supernatants by binding to Con A-sepharose-4B (Fig. 7). This demonstrates that the failure of the immunoprecipitation of E-cad100 with the monoclonal antibody was due to its conformation specificity and not due to proteolytic degradation that is dependent on the Ca^{2+} concentration.

V. Inhibition of the reassembly of intercellular junctions of A6 cells by mAbs to *Xenopus* E-cadherin.

To determine whether mAbs to *Xenopus* E-cadherin could interfere with its adhesion function in cultured cells, a resistance recovery assay for measuring the sealing of the tight junction was used (34). The assay was modified slightly from the original method so that it could be used on the amphibian A6 cells. Intercellular junctions were opened by incubation in Ca^{2+} free medium for 6.5 min at 28°C, and cells were treated with various antibodies for 40 min on ice. After returning the monolayers to Ca^{2+} containing medium at 28°C, the recovery of transepithelial electrical resistance was followed over time as a measure of the rate of resealing of tight junctions. All three of the anti-E-cadherin mAbs tested strongly inhibited the recovery of transepithelial resistance (Fig. 8). A mixture of the three mAbs

inhibited more strongly than the individual mAbs. Therefore, the protein recognized by these mAbs functions in the formation of intercellular contacts, as does E-cadherin in MDCK cells (36).

VI. Expression of E-cadherin in other epithelial tissues.

The E-cad100 for the production of mAbs was purified from the A6 kidney epithelial cell line. To determine whether E-cadherin is expressed in other epithelial tissues, extracts of lung, liver, and brain of adult *Xenopus laevis* were analyzed by immunoblotting with a mixture of anti-E-cadherin mAbs (Fig. 9). High levels of E-cadherin expression were observed in A6 cells, lung, and liver (lane a, c, d), but very little was detected in brain (lane b). The intact 140 kD E-cadherin molecule and its degradation products were detected in both lung and liver. More extensive proteolysis seems to occur during extraction of these tissues than during the extraction of A6 cells. Nonimmune antibody did not react with any of these polypeptides (lane a'-d').

Immunofluorescence staining of A6 cells and cryostat sections of tissues with anti-E-cadherin mAbs are shown in Figure 10. In A6 cells, mAb 19A2 stained the basolateral plasma membrane (Fig. 10 A). Hepatocytes were also stained at the basolateral plasma membrane (Fig. 10 C). It was not easy to discern the apical plasma membrane facing a small bile canalicus in these thick sections. Epithelial cells of the lung were stained with mAbs against E-cad100 (Fig. 10 E). Again, staining was localized at the basolateral plasma membrane. Endothelial cells were not stained. Nonimmune antibody did not stain (Fig. 10 B, D ,F)

Discussion

The monoclonal antibodies raised against *Xenopus* E-cadherin enabled us to study the role of E-cadherin in *Xenopus laevis* biochemically, immunocytochemically, and functionally. Two mAbs (8C2 and 31D2) were able to distinguish the Ca^{2+} dependent conformation of E-cadherin. This property of mAb 8C2 may be useful in the future to analyze the functional significance of the conformational change in this molecule during intracellular transport or during embryonic development. *Xenopus* E-cadherin was first synthesized as a higher molecular mass precursor as in other species (29, 73), (M. Wheelock, unpublished observation; T. Chernov-Rogen and B. Gumbiner, unpublished observation). Slow conversion of the precursor into the mature form of E-cadherin is probably due to the low growth temperature of the A6 cells (28°C), which may result in the accumulation of the precursor to a detectable level even in the immunoblotting assay. Like E-cadherin in all species, this protein in *Xenopus laevis* was expressed at high levels in epithelial tissues such as liver and lung epithelia, but not in brain or endothelia. As expected for an adhesion molecule, *Xenopus* E-cadherin was localized to the basolateral plasma membrane domain in different epithelial cell types. Finally, the functional criterion for being an adhesion molecule was satisfied by the ability of anti-E-cadherin mAbs to inhibit the formation of cell junctions between cultured epithelial cells. Thus, the mAbs

generated in this study can be considered to be specific probes for E-cadherin in *Xenopus laevis*.

Figure 1. Determination of serum titers of immunized mice. Two Balb/C mice were immunized twice with ~5 μg of purified E-cad100. The titers are checked by immunoblotting of E-cad100 with the mouse serum at a 100 fold dilution (lane a and d) and at a 1,000 fold dilution (lane b and e) or with a preimmune serum at a 100 fold dilution (lane c and f). Numbers at left are molecular weight markers in kilodaltons.

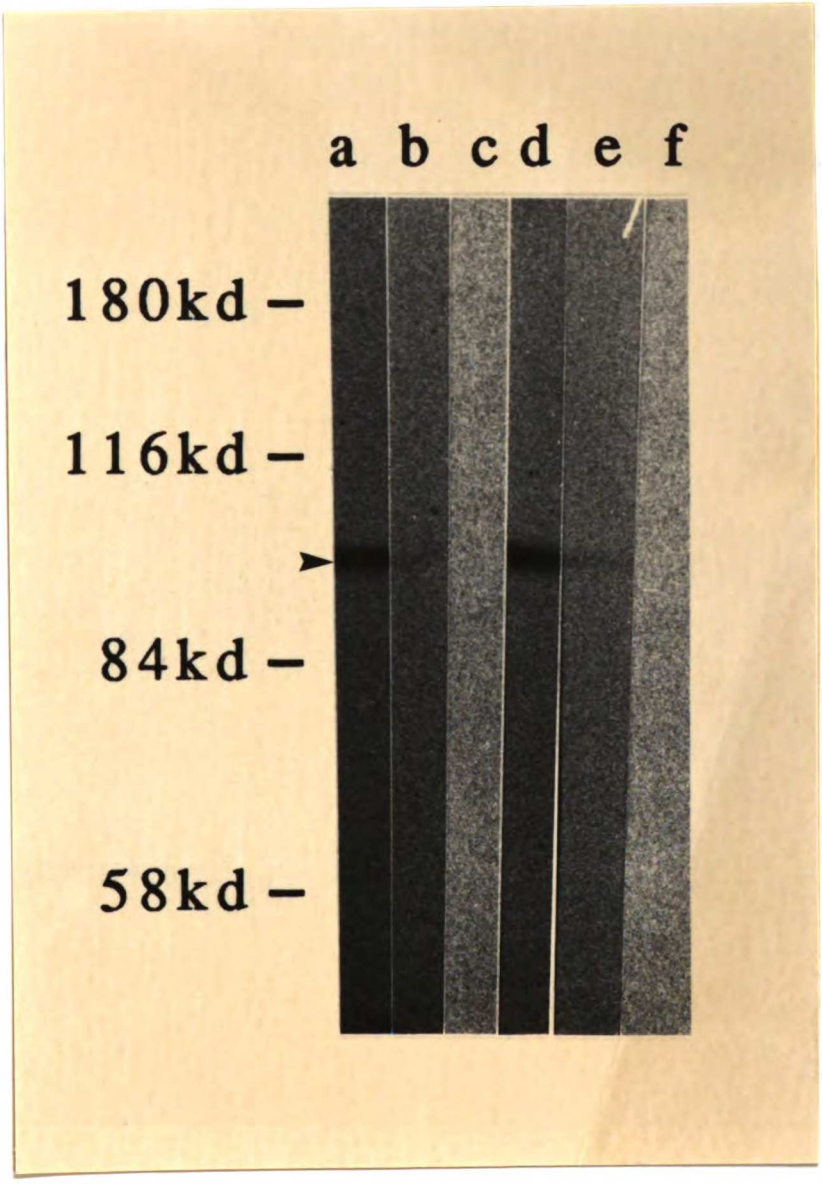

a b c d e f
180kd –
116kd –
84kd –
58kd –

Figure 2. Immunoblotting of SDS extracts of A6 cells with monoclonal antibodies to *Xenopus* E-cadherin. $2x10^5$ A6 cells for each lane were extracted with boiling SDS extraction buffer and immunoblotted with the hybridoma supernatants of mAbs 5D3 (lane a), 8C2 (lane b), 19A2 (lane c), 31D2 (lane d), and 15B2 (lane e). Numbers at left are molecular weight markers in kilodaltons.

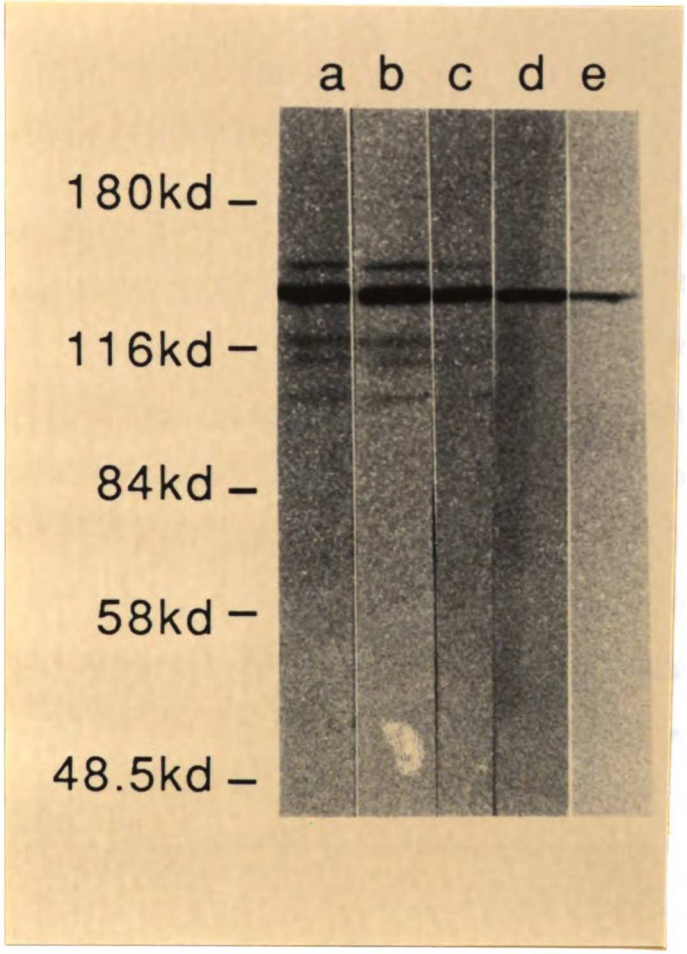

a b c d e
180kd
116kd
84kd
58kd
48.5kd

Figure 3. Characterization of E-cadherin with the monoclonal antibodies. A. The phase-partitioning of E-cadherin in the Triton X-114 extracts of A6 cells. The equivalent fractions of the aqueous (lane c), the detergent (lane a), the insoluble pellet phase (lane b) were immunoblotted with a mixture of monoclonal antibodies (5D3, 8C2, and 19A2). B. Tryptic conversion of E-cadherin in solution. 2×10^5 A6 cells were extracted with 1 % Triton X-114 in solution A in the absence (lane c) or in the presence (lane b) of protease inhibitors, and the aqueous phases were immunoblotted. The sample in lane c was trypsized (lane d). The same number of A6 cells were extracted with boiling SDS buffer (lane a) to compare the extent of degradation during the two different extraction procedures. Numbers at left are molecular weight markers in kilodaltons.

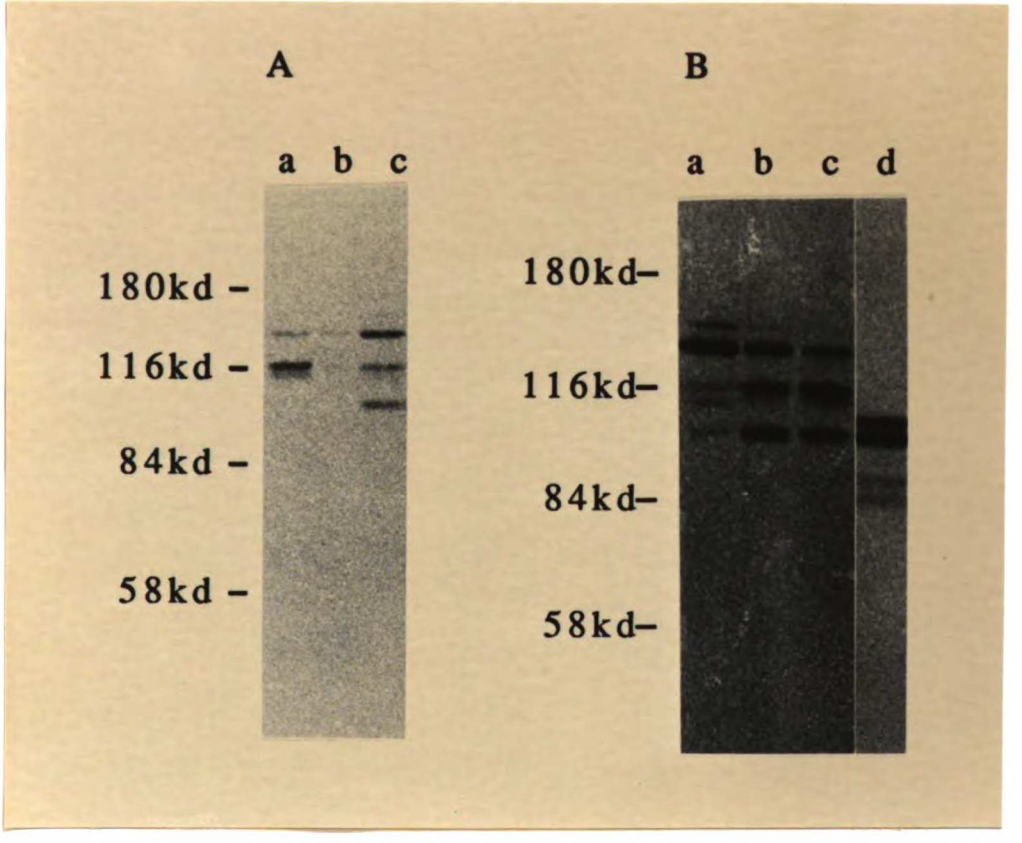

A
a b c
180kd –
116kd –
84kd –
58kd –
B
a b c d
180kd–
116kd–
84kd–
58kd–

Figure 4. Identification of the 155 kD precursor to the 140 kD mature E-cadherin. A6 cells were pulse labelled with ^{35}S-methionine for 10 min, and then chased for the indicated times. Cell extracts were immunoprecipitated with the mixture of mAbs 5D3, 8C2 and 19A2, and analyzed by SDS PAGE and fluorography. Numbers on the left side are molecular weight markers in kD. Numbers on the right side are the calculated molecular weight in kilodaltons of the precursor (155 kD), mature E-cadherin (140 kD), its degradation product (116 kD), and its associated protein (100 kD).

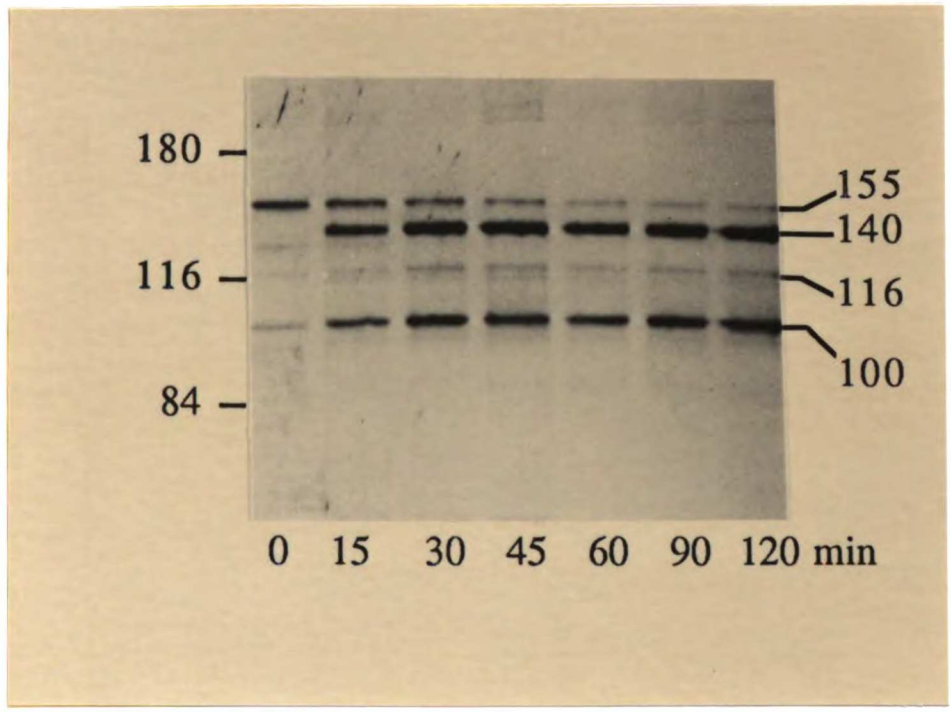

180
116
84
155
140
116
100
0 15 30 45 60 90 120 min

Figure 5. Immunoprecipitation of ^{35}S-methionine labelled A6 cells with the monoclonal antibodies to E-cadherin. A6 cells were labelled with 0.3 mCi of ^{35}S-methionine overnight. The supernatants of whole cell trypsin digests were immunoprecipitated with monoclonal antibodies 5D3, 8C2, and 19A2 respectively, analyzed by SDS-PAGE, and followed by fluorography. For comparison of molecular weight of canine E-cadherin, MDCK cells were labelled and immunoprecipitated with a monoclonal antibody rr1. (+) and (-) at the bottom denote trypsin digests in the absence (-) or in the presence (+) of Ca^{2+}. Numbers on the left side are molecular weight markers in kilodaltons.

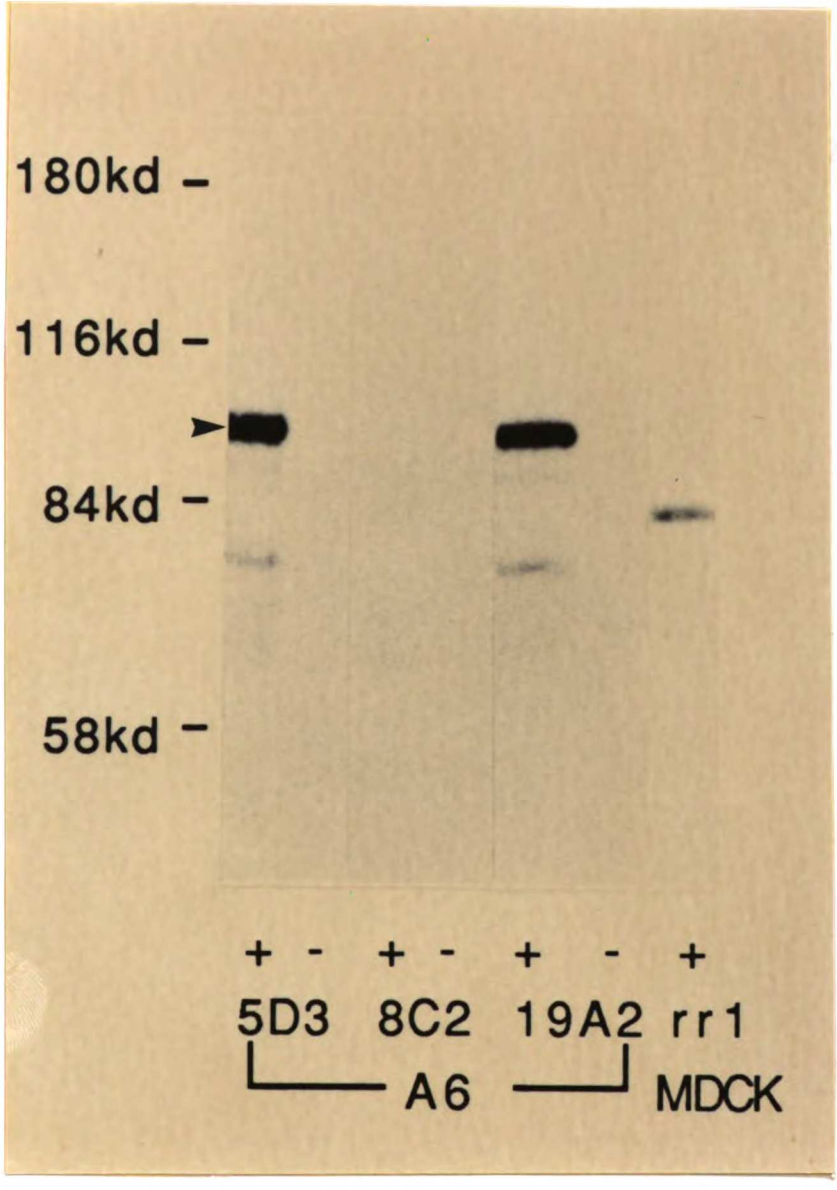

180kd
116kd
84kd
58kd
+ - + - + - +
5D3 8C2 19A2 rr1
A6
MDCK

Figure 6. Conformation specificity of mAbs against *Xenopus* E-cadherin. ^{35}S-methionine labelled E-cad100 in the absence of Ca^{2+} (-), or in the presence of Ca^{2+} (+) was immunoprecipitated with mAbs 5D3, 19A2, 15B2, 31D2, and 8C2, and analyzed by SDS-PAGE and fluorography. Arrow head = E-cad100. Numbers on the left side are molecular weight markers in kilodaltons.

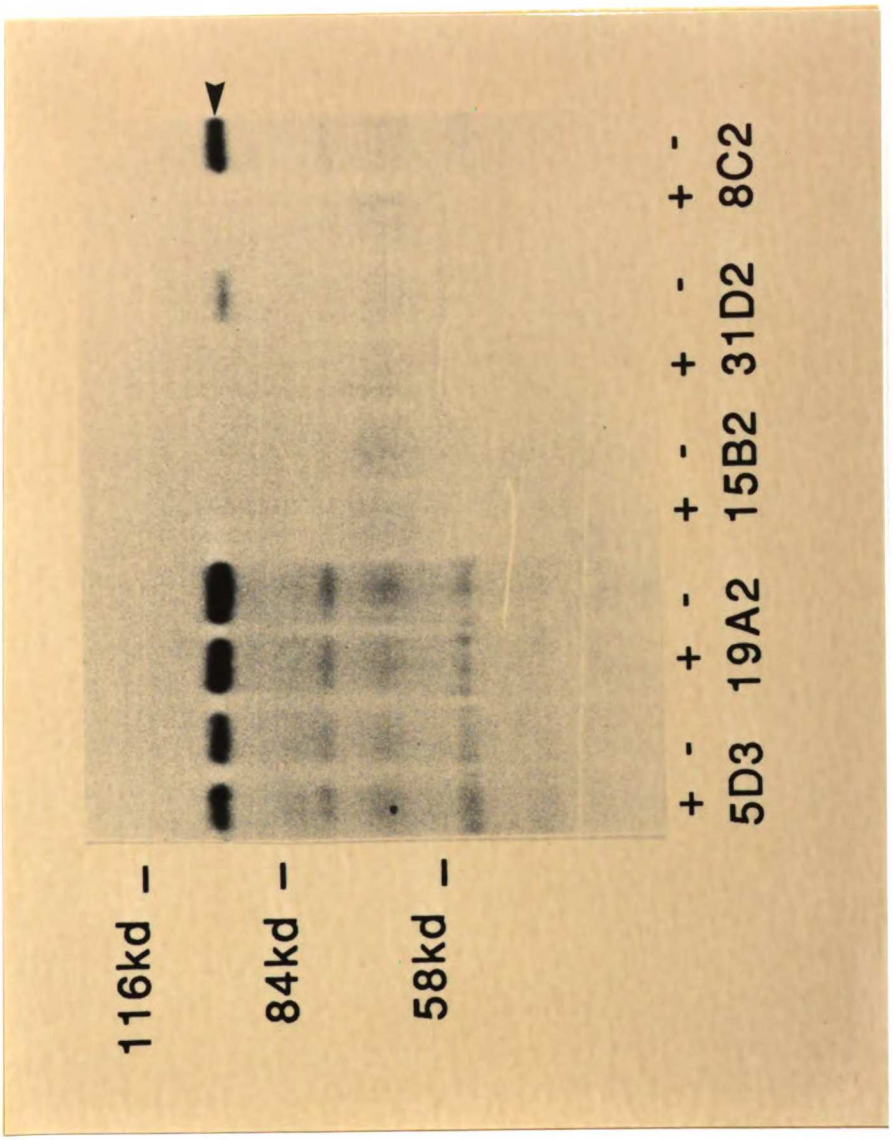
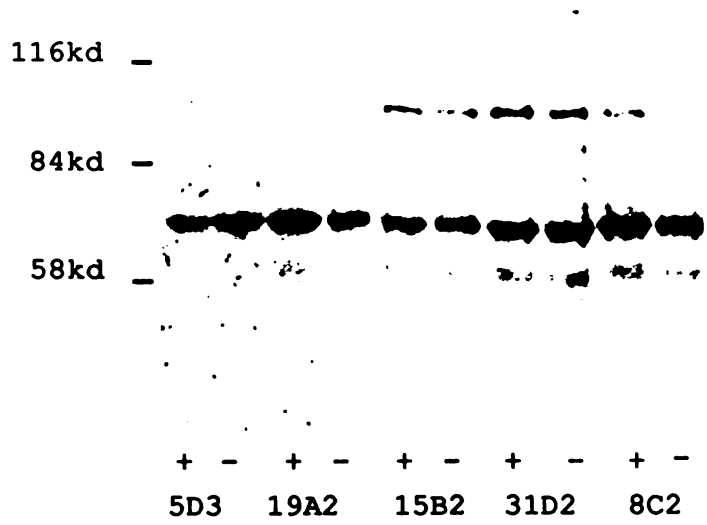

116kd –
84kd –
58kd –
+ - + - + - + - + -
5D3 19A2 15B2 31D2 8C2

Figure 7. The recovery of unbound E-cad100 by binding to Con A sepharose-4B. The glycoproteins remaining in the supernatants after immunoprecipitation with individual monoclonal antibodies were retrieved by binding to Con A beads. (+) and (-) at the bottom denote trypsin digests in the absence (-) or in the presence (+) of Ca^{2+}. Numbers on the left side are molecular weight markers in kilodaltons.


116kd
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5D3 19A2 15B2 31D2 8C2

Figure 8. Inhibition of tight junction formation between A6 cells by mAbs to *Xenopus* E-cadherin. Monolayers of A6 cells grown on filters were incubated in Ca^{2+} free medium at 28°C. At 6.5 min, they were then treated with mAbs 5D3 (-○-), 19A2 (··□···), 8C2 (--△--), a mixture of mAbs 5D3, 19A2, and 8C2 (-■-), or control mAb rr1 (-●-) for 40 min on ice. The recovery of transepithelial resistance was measured at the indicated times after transfer into the recovery medium at 28°C. Values are mean + SEM, n=3

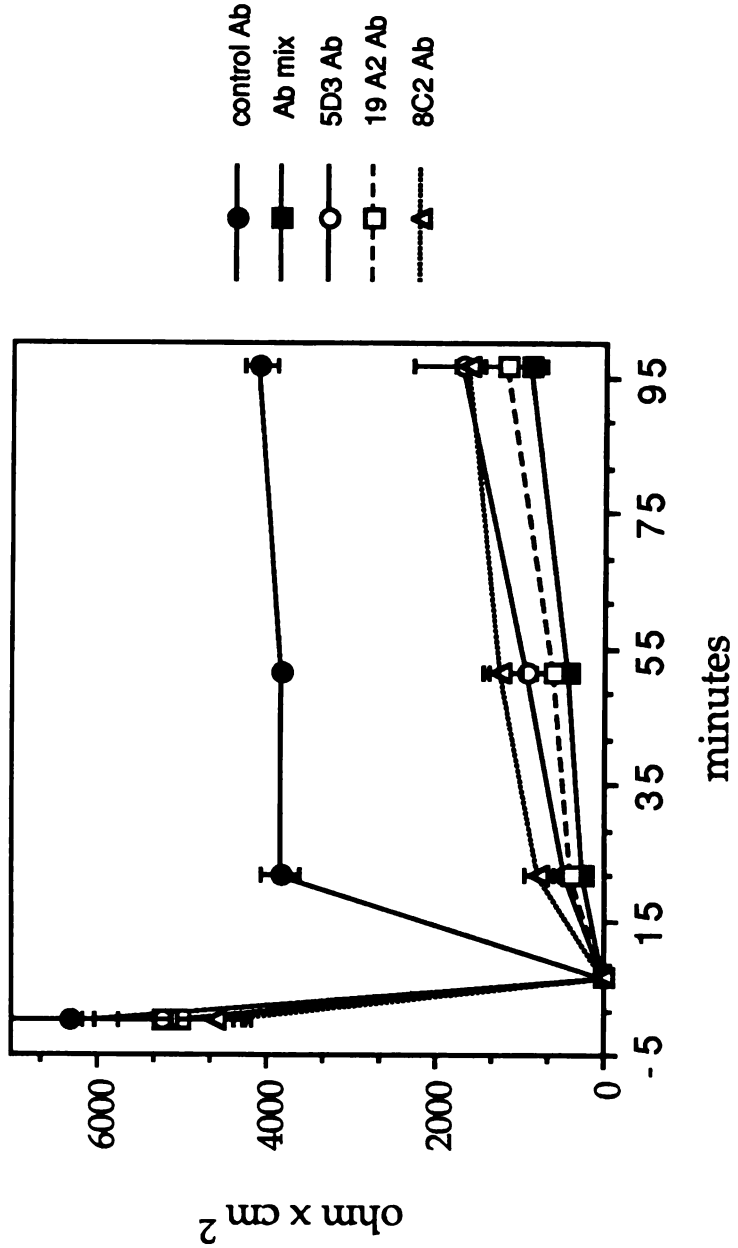

control Ab
Ab mix
5D3 Ab
19 A2 Ab
8C2 Ab
ohm x cm 2
6000
4000
2000
0
-5
15
35
55
75
95
minutes

Figure 9. Expression of *Xenopus* E-cadherin in epithelial tissues. Extracts of cultured A6 cells, brain, liver, and lung from *Xenopus laevis*, all containing the same amounts of protein, were concentrated by binding to Con A-sepharose, and immunoblotted with the mixture of mAbs 5D3, 8C2, and 19A2 (lane a-d), or with nonimmune antibody for the control (lane a'-d'). All were then reacted with alkaline phosphatase conjugated anti-mouse IgG. Numbers on the left side are molecular weight markers in kilodaltons.

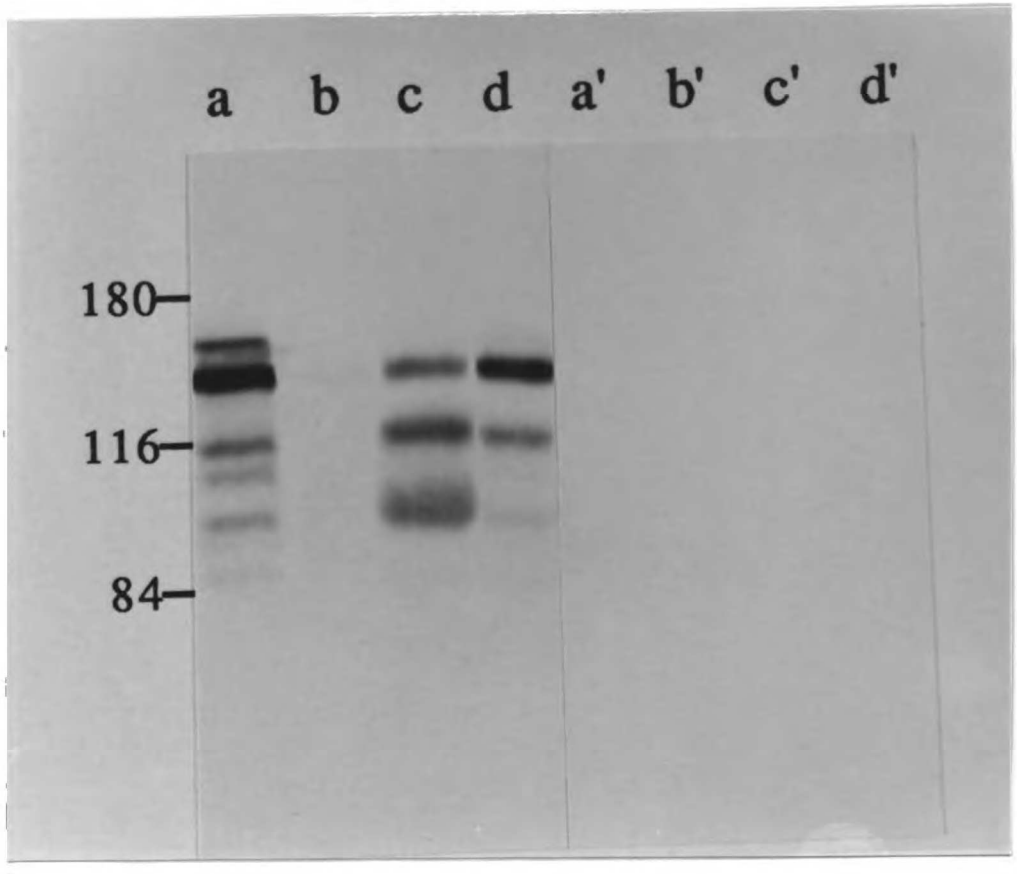
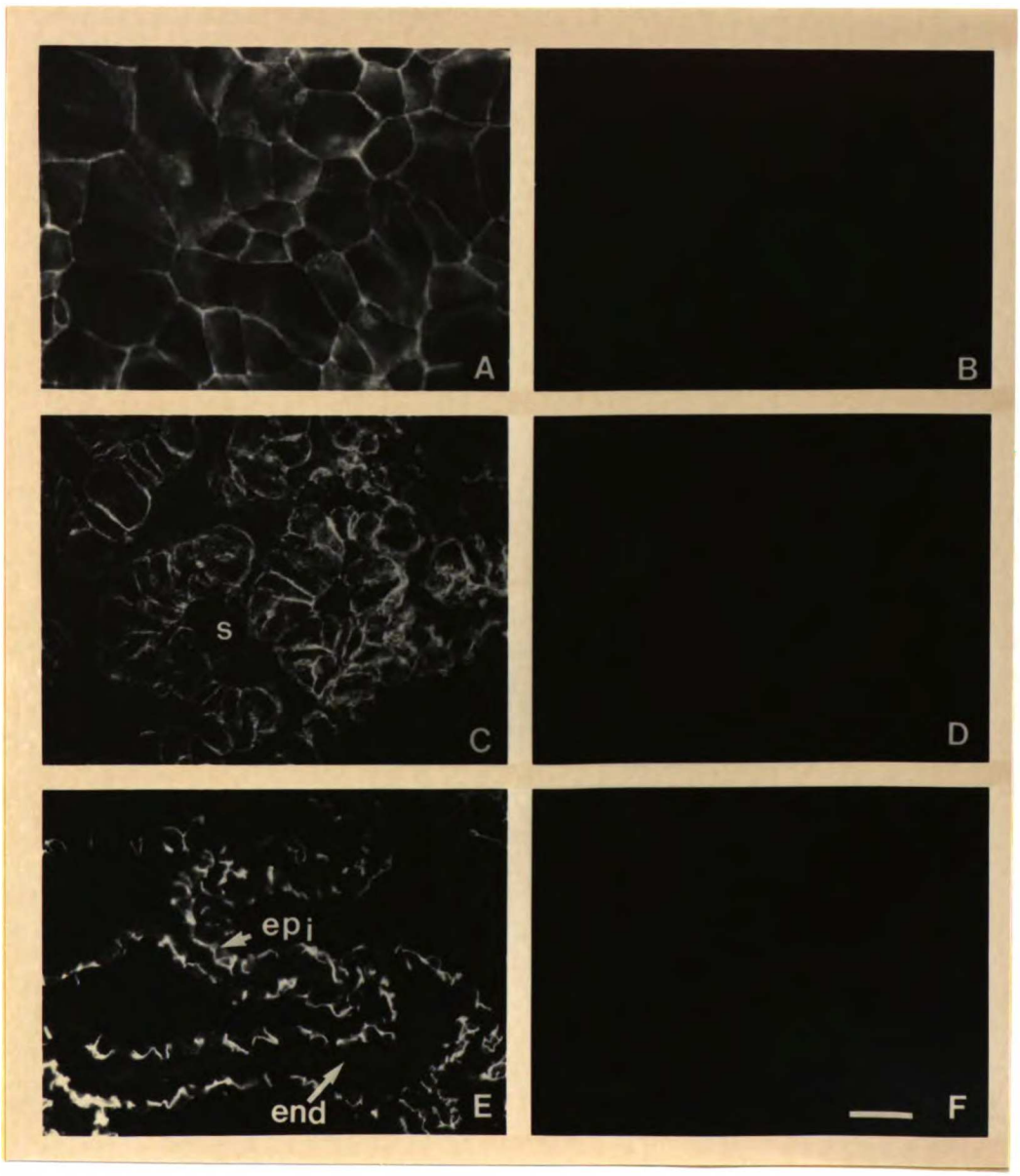

a
b
c
d
a'
b'
c'
d'
180
116
84

Figure 10. Immunocytochemistry of E-cadherin in A6 cells and epithelial tissues. A6 cells fixed with methanol and acetone were stained with mAb 19A2 (A), or with nonimmune antibody as the control (B). Cryostat sections (16 μm) of liver (C, D), and lung (E, F) fixed with 95% ethanol, were stained with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2 (C, E), or with nonimmune antibody as the control (D, F). Sinusoid (s), alveolar epithelial cells (epi), endothelial cells (end). Bar = 40 μm.


A
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Chapter 4

Expression of E-cadherin in Early *Xenopus* Embryos

Summary

The expression of the Ca^{2+}-dependent epithelial cell adhesion molecule E-cadherin in the early stages of embryonic development of *Xenopus laevis* was examined. The time of E-cadherin expression in early *Xenopus* embryos was determined by immunoblotting. Unlike its expression in early mouse embryos, E-cadherin was not present in the eggs or early blastula of *Xenopus laevis*. These findings indicate that a different Ca^{2+}-dependent cell adhesion molecule, perhaps another member of the cadherin gene family, is responsible for the Ca^{2+}-dependent adhesion between cleavage stage *Xenopus* blastomeres. Detectable accummulation of E-cadherin started just prior to gastrulation at stage 9 1/2, and increased rapidly up to the end of gastrulation at stage 15. In stage 15 embryos, specific immunofluorescence staining of E-cadherin was discernible only in ectoderm, but not in mesoderm or endoderm. The ectoderm at this stage consists of two cell layers. The outer cell layer of ectoderm was stained intensely and staining was localized to the basolateral plasma membrane of these cells. Lower levels of staining was observed in the inner cell layer of ectoderm. At the neurula stage, the decrease in E-cadherin expression was observed in the neural fold as seen in the other species. The coincidence of E-cadherin expression with the process of gastrulation and its restriction to the ectoderm indicate that it may play a role in the morphogenetic movements of gastrulation and resulting segregation of embryonic germ layers.

Introduction

In order to better understand the developmental significance of the expression of E-cadherin (and other cadherins), it will be important to investigate how E-cadherin influences or regulates the behavior of cells in epithelia undergoing morphogenesis. In the earliest stages of cleaving embryos, a multicellular blastula is formed, which is held together by a Ca^{2+}-dependent adhesion mechanism (62, 64, 65) and is physiologically separated from the bathing medium by a tightly sealed surface epithelium (60, 76). Up until the midblastula transition, cell division is reductive (53), and the cells are built largely from components stored in the egg (4, 85, 99, 101). The cleavage furrows and resulting plasma membranes in these early embryos seem to be assembled from exocytosis of vesicles stored in the egg (4). Thus, the assembly of cell contacts and junctions is very extensive, synchronous, and rapid during these stages. Amphibian embryos have also been successful experimental models for the study of cell adhesion and cell behavior in morphogenetic events such as gastrulation (31). In classical embryology, early amphibian embryos were used to demonstrate the importance of selective cell adhesion in morphogenesis (88, 92). More recently *Xenopus* embryos have been used to study the developmental regulation of gene expression and the molecular biology of tissue induction and differentiation (18).

E-cadherin is the earliest adhesion molecule expressed in mouse and chicken embryos (39, 91, 93). Therefore, I asked whether it might be

stored in vesicles in the *Xenopus* egg, inserted into the plasma membrane during cleavage, and responsible for the Ca^{2+}-dependent adhesion between early blastomeres. Also, in order to begin to investigate the influence of E-cadherin on cell behavior and its role in the morphogenesis of epithelial sheets, I examined whether changes in the levels or location of its expression were associated with the formation of the primary germ layers (ectoderm, mesoderm, and endoderm) during gastrulation.

Results

I. Expression of E-cadherin just before gastrulation.

To determine whether E-cadherin is stored in eggs, and involved in the Ca^{2+}-dependent adhesion of early blastomeres and/or later developmental processes of *Xenopus* embryos, its time of expression was examined. Eggs and embryos at different stages were extracted with an NP-40 containing buffer. The glycoprotein fraction was enriched by binding to Con A-sepharose-4B and immunoblotted with anti-E-cadherin mAbs (Fig. 1). Detectable accumulation of E-cadherin started at 10 hr after fertilization (stage 9 1/2 as defined by Nieuwkoop (63)) and increased rapidly up to 24 hr (stage 15). The 155 kD precursor, the 140 kD mature protein and degradation products, which had been observed in A6 cells, were also detected in embryos. The lack of detection of E-cadherin prior to stage 9 1/2 was probably not due to difficulties with extraction by NP-40. The extractability of E-cadherin by NP-40 (Fig. 1, 24 hr*) from embryos after stage 9 1/2 was comparable to extraction with the harsher conditions using a mixture of SDS, deoxycholate and Triton X-100 (Fig 1, 24 hr**). Nor could the lack of detection be attributable to using the Con A enrichment step prior to SDS-PAGE. Enrichment by immunoprecipitating with a combination of the mAbs and subsequent immunoblotting gave the same result (compare 24 hr and 24 hr* in Fig. 1).

To determine whether E-cadherin might be stored in eggs at a lower concentration, 2,000 eggs (20 times more eggs than that used in Figure 1) were extracted with the NP-40 containing buffer. Extracts were immunoprecipitated either with a mixture of mAbs 8C2 , 5D3 and 19A2 or with individual mAbs, and then immunoblotted with either the individual mAbs (Fig. 2 , lane d-g) or the mixture (Fig. 2, lane a-c). No specific E-cadherin polypeptide was detected, even when the blot was overdeveloped. Therefore, by the criteria of binding of these four different mAbs, E-cadherin did not seem to be present in eggs of *Xenopus laevis*.

II. Spatial localization of E-cadherin expression

The spatial localization of E-cadherin expression was examined by immunofluorescence staining of cryostat sections of embryos with a mixture of mAbs 8C2 , 5D3 and 19A2. In stage 15 embryos, specific staining was discernable only in ectoderm, but not in mesoderm and endoderm (Fig. 3 B). The ectoderm at this stage consists of two cell layers. The outer cell layer of ectoderm was stained with mAbs, of which staining was exclusively at the basolateral plasma membrane (Fig. 3 C). At higher magnification, the high level of the basolateral staining of E-cadherin in embryos always coincided with the presence of cortical pigment granules (Fig. 3 D), which is a good marker for the outer cell layer of ectoderm (R. Keller, personal communication). A lower level of staining was observed in the inner cell layer of the ectoderm (Fig. 3 B, C). With nonimmune antibody as a control, the

ectoderm was not stained and only background fluorescence was observed (Fig. 3 A).

No specific staining was ever observed in any internal cell layers at stage 15. Very low levels of expression of E-cadherin in the mesoderm and endoderm cannot be excluded by these experiments. Low levels of staining would not be detectable above the background autofluorescence from yolk platelets. It was not possible to discern specific staining in any cells of the embryo at stage 9 1/2 and 10 1/2 (not shown). E-cadherin expression levels at this stage were probably still too low to be detected by this method. Again, autofluorescence from yolk platelets increased the background and probably interfered with the detection of low levels of staining.

III. Decrease of E-cadherin expression in the neural fold.

A decrease in E-cadherin expression during neural induction is observed in many species (39, 91) and was also observed in one experiment with *Xenopus* embryos. Embryos were prefixed with 3 % TCA and embeded in sucrose/gelatin before cryostat sectioning. This procedure preserved well the internal structure of embryos. Embryos at stage 17 (mid-neurula) were stained with a mixture of anti-E-cadherin antibodies. Expression of E-cadherin was reduced in the neural fold (Fig 4). In this study, it was not determined whether decrease of E-cadherin expression is due to the proteolytic degradation or dilution of pre-existing E-cadherin upon cell division, after transcriptional shutdown of the E-cadherin gene. Experiments

addressing this question may be possible after the gene encoding *Xenopus* E-cadherin is cloned.

IV. Effect of monoclonal antibidies to E-cadherin on gastrulation of *Xenopus* embryos.

To test the functional role of E-cadherin in gastrulation, a highly concentrated mixture of three purified mAbs (5D3, 8C2 and 19A2) was microinjected into the blastocoel cavity of embryos at stage 8. No obvious effect on subsequent development was observed by simple morphological examination with a dissecting stereomicroscope. Cryostat sections of injected embryos showed that antibodies seemed to have access to the ectodermal layers. Gastrulation is a complex event that proceeds through a concerted action of many different regional cell movements (31). Therefore explants of isolated regions of a blastula or gastrula, which have been shown to undergo region specific morphogenetic movements (52), may be more useful for examining the functional activity of E-cadherin using antibody perturbation. Future experiments involving tissue explants and examination of more subtle changes may reveal a perturbation of some component of gastrulation.

Discussion

Although blastomeres in early cleavage stage *Xenopus* embryos form an epithelial-like tightly sealed blastula (60, 76) and are held together by a Ca^{2+}-dependent adhesion mechanism (62, 64, 65), E-cadherin appeared not to be involved in cell-cell interactions in the early *Xenopus* blastula. It began to accumulate to detectable levels only at stage 9 1/2, just prior to gastrulation. E-cadherin did not seem to be stored in the egg, unlike many components required for the assembly of cell organelles in cleavage stage *Xenopus* embryos (4, 85, 99, 101). It was undetectable in extracts of a large number of eggs. The use of four independent mAbs to assay E-cadherin makes it very unlikely that E-cadherin went undetected simply because of a masked or missing epitope. These findings suggest that another Ca^{2+}-dependent adhesion molecule, perhaps another member of the cadherin gene family (90), is responsible for the adhesion between cleavage stage *Xenopus* blastomeres and in mediating the assembly of tight epithelial junction between surface blastomeres (see chapter 5).

In contrast to our findings, a previous publication on *Xenopus* embryos using antibodies raised to chicken L-CAM, concluded that L-CAM or E-cadherin was expressed in most, if not all of the early blastomeres of *Xenopus laevis* (55). However, no biochemical analysis of E-cadherin levels in early embryos was reported in that study. Also, the antibodies to chicken L-CAM stained diffusely the cytoplasm of both embryonic cells and adult hepatocytes. Diffuse cytoplasmic

staining, while not impossible, is inconsistent with the known functions and subcellular distributions of the cadherins. Our finding of a later time of expression is supported both by a biochemical analysis of E-cadherin levels and by localization at the basolateral plasma membrane, a location typical of many other adhesion molecules. The reported staining of early blastomeres with anti-chicken L-CAM may have been due to nonspecific reactivity of the antibodies on *Xenopus* tissues.

This late expression of E-cadherin in *Xenopus laevis* contrasts with its expression in the mouse and chick embryos. E-cadherin is present on the surface of the mouse egg, is already being synthesized in two-cell mouse embryos (93), and functions in compaction at the 8-16 cell stage to form the epithelial trophectoderm of the blastocyst (17, 83, 94). E-cadherin is also expressed in the inner cell mass, which developes into the embryo proper, prior to gastrulation (16, 93). In chick embryos, L-CAM was found at low levels in all cells of the blastoderm in pregastrulation embryos (91). These differences between *Xenopus*, mouse, and chick suggest that there is no set developmental order for the expression of cell adhesion molecules for all vertebrate species. Perhaps, N-cadherin or P-cadherin, which are usually expressed later in the development of mouse and chick (40), mediate the Ca^{2+}-dependent adhesion between early blastomeres in *Xenopus*. Alternatively, a novel cadherin or a new kind of Ca^{2+}-dependent adhesion molecule could be responsible for intercellular adhesion in cleavage stage *Xenopus* embryos (see chapter 5).

Expression of the E-cadherin protein in *Xenopus* embryos was first observed at stage 9 1/2, just prior to the start of gastrulation. We do not yet know whether its expression is controlled by the onset of embryonic transcription at the midblastula transition (53) or by the post-transcriptional activation of stored maternal messenger RNAs as occcurs with fibronectin (54) and nuclear lamin synthesis (89). By the end of gastrulation, E-cadherin accumulated to the greatest extent in the outer cell layer of the ectoderm. At this time, the ectoderm consists of two cell layers. A lower level of immunofluorescence staining was noticed in the inner cell layer of the ectoderm. It is not yet possible from our experiments to completely rule out even lower levels of E-cadherin expression by cells of the endoderm or the mesoderm. The background level of fluorescence from embryonic cells may have been great enough to prevent the detection of very low amounts of the protein. In fact, we were unable to detect E-cadherin by immunofluorescence staining in the early gastrula even though small amounts were demonstrated biochemically in immunoblotting experiments. The high level of E-cadherin expression by the ectoderm does suggest, however, that E-cadherin might play a significant role in the morphogenesis of this tissue during gastrulation.

The pattern of E-cadherin expression in the primary germ layers during gastrulation in *Xenopus* embryos differs from mouse and chick embryos. In *Xenopus* embryos, E-cadherin becomes expressed only in the ectoderm during gastrulation. In mouse embryos, E-cadherin is expressed in the endoderm as well as the ectoderm (16). During gastrulation of chick embryos, expression of L-CAM persists in the

ectoderm, but disappears from mesodermal and definitive endodermal cells as they separate from the ectoderm during ingression (91). These differences in the pattern of E-cadherin expression may reflect the different kinds of cell behaviors involved in the formation of the germ layers during gastrulation in various species.

Previous studies of *Xenopus* gastrulation suggest two possible (and perhaps related) roles for E-cadherin in the morphogenesis of the ectoderm. In one series of experiments, Keller and colleagues have shown that epithelial cells undergo region specific behaviors during gastrulation (31) that could involve different regional forms of intercellular contact. For example, cells in the involuting marginal zone are highly motile with respect to one another and undergo extensive rearrangements despite the fact that they remain in intimate contact and tightly sealed (51, 52). In contrast, the animal cap, which gives rise to ectoderm, undergoes epiboly, a process of isotropic expansion of the epithelium in which the cells flatten and spread. Perhaps E-cadherin expression at the cell surface is associated with epibolic movement and/or incompatible with cellular rearrangements. In this respect, it is interesting that a decrease in E-cadherin expression has been noted in epithelia undergoing extensive morphogenetic movements, such as the invagination of the ectoderm during formation of the chicken and mouse neural tube (67, 91).

Another important role for E-cadherin in gastrulation is suggested by the hypothesis that the cadherins are responsible for the differential cell adhesion that leads to the sorting out of cell types in developing tissues (66, 90). In amphibian embryos, differential cell adhesion is

believed to play a role in the segregation of the embryonic germ layers (88, 92). Interestingly, recent experiments have shown that animal cap cells isolated from *Xenopus laevis* become able to recognize and migrate preferentially into the ectoderm only when they are derived from embryos after stage 10 (47). Perhaps this onset in the ability of animal cap cells to recognize the ectoderm is mediated by the expression of E-cadherin, which normally commences at this same time.

It should now be possible to test directly whether either of these two ideas about the function of E-cadherin in *Xenopus* gastrulation is correct. The use of specific probes for adhesion molecules in this experimentally accessible developmental system holds exciting prospects for learning about the molecular and cellular processes controlling the morphogenesis of tissues.

Figure 1. Expression of E-cadherin in *Xenopus* embryos. 150-200 eggs or embryos for each sample were extracted with NP-40 containing buffer at the indicated times after fertilization (0-24 hr). The glycoprotein fractions were enriched by binding to Con A-sepharose and immunoblotted with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2. The embryos at 24 hr were extracted either with NP-40 containing buffer (24*) or immunoprecipitation buffer (24**). Both extracts were immunoprecipitated with the mixture of the monoclonal antibodies and immunoblotted with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2. A6 = an extract of A6 cells for comparison. Numbers on the left side are molecular weight markers in kilodaltons.

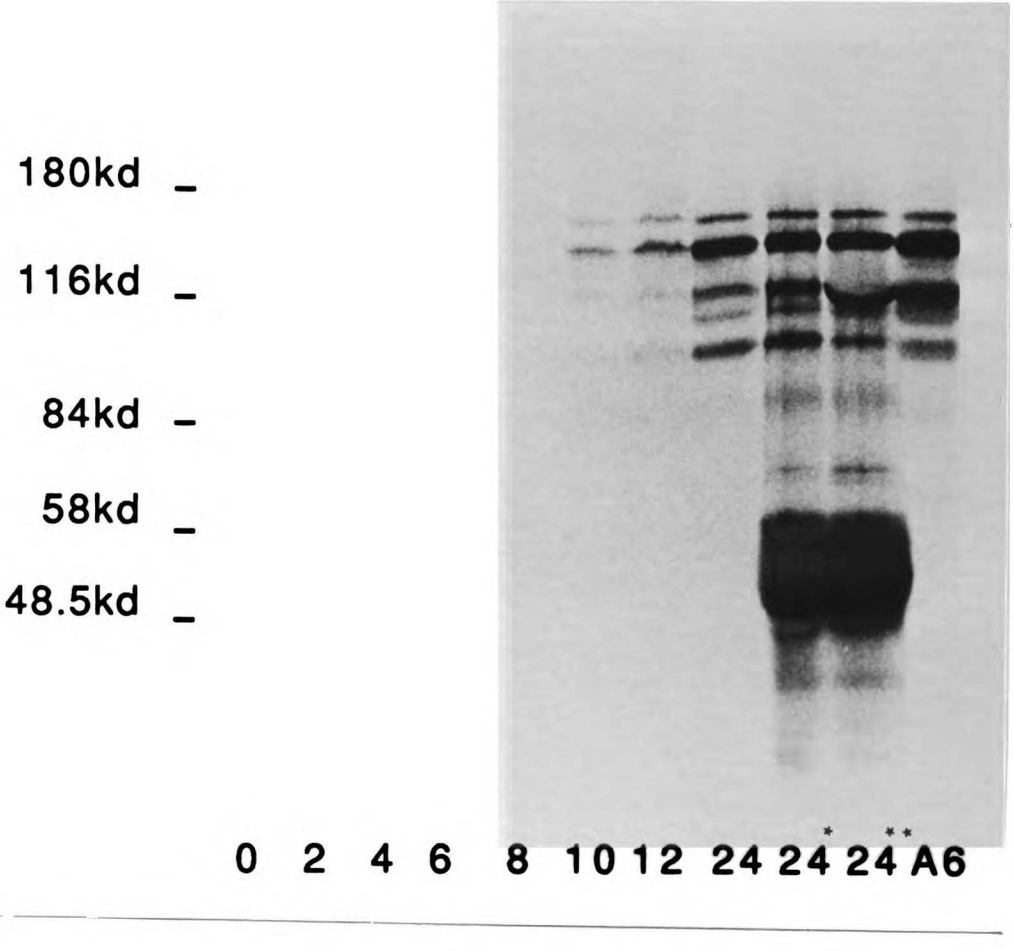
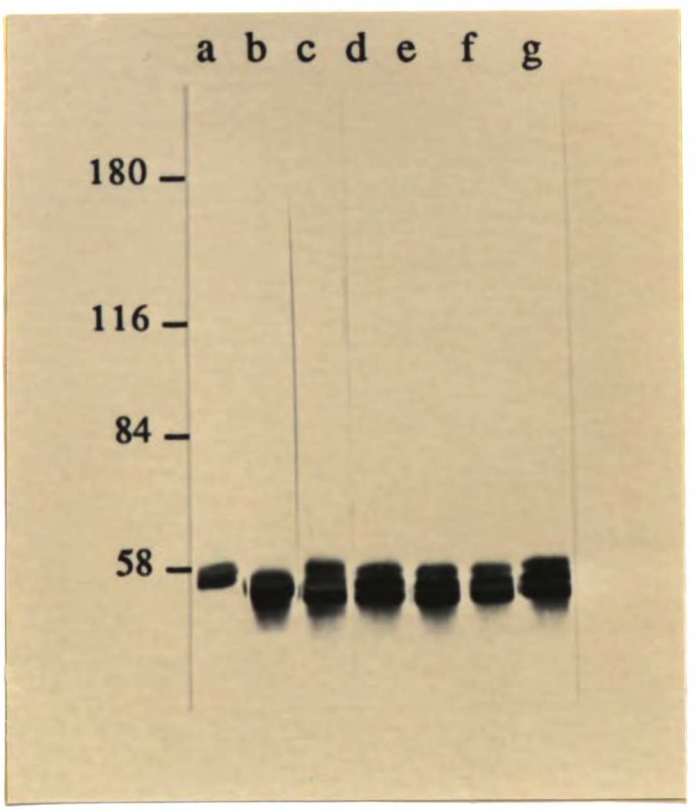

180kd
116kd
84kd
58kd
48.5kd
0 2 4 6 8 10 12 24 24* 24** A6

Figure 2. Attempt to detect E-cadherin in *Xenopus* eggs. 2,000 eggs for each lane were extracted with immunoprecipitation buffer, immunoprecipitated with mAbs 5D3 (lane a), 8C2 (lane b), 19A2 (lane c), and immunoblotted with the mixture of mAbs 5D3, 8C2, 19A2, and 31D2. The same samples were immunoprecipitated with the mixture of mAbs (lane d-g), and immunoblotted with individual mAb 5D3 (lane d), 8C2 (lane e), 19A2 (lane f), and 31D2 (lane g). The ~ 55kD bands are heavy chains of mouse IgGs used for immunoprecipitation. Numbers on the left side are molecular weight markers in kilodaltons.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58

Figure 3. Localization of E-cadherin in the ectoderm of *Xenopus* embryos. Indirect immunofluorescence staining of transverse cryostat sections (16 μm) of stage 15 embryos. A. Background staining control by nonimmune antibody. B. C. and D. Staining with a mixture of mAbs 5D3, 8C2, 19A2, and 31D2. ec (ectoderm), ms (mesoderm), en (endoderm), ar (archenteron), ol (outer layer of ectoderm), il (inner layer of ectoderm), and pg (cortical pigment granule). A, B, and C are the same magnification. Bar = 40 μm. D. Higher magnification of ectodermal staining. Bar = 40 μm.

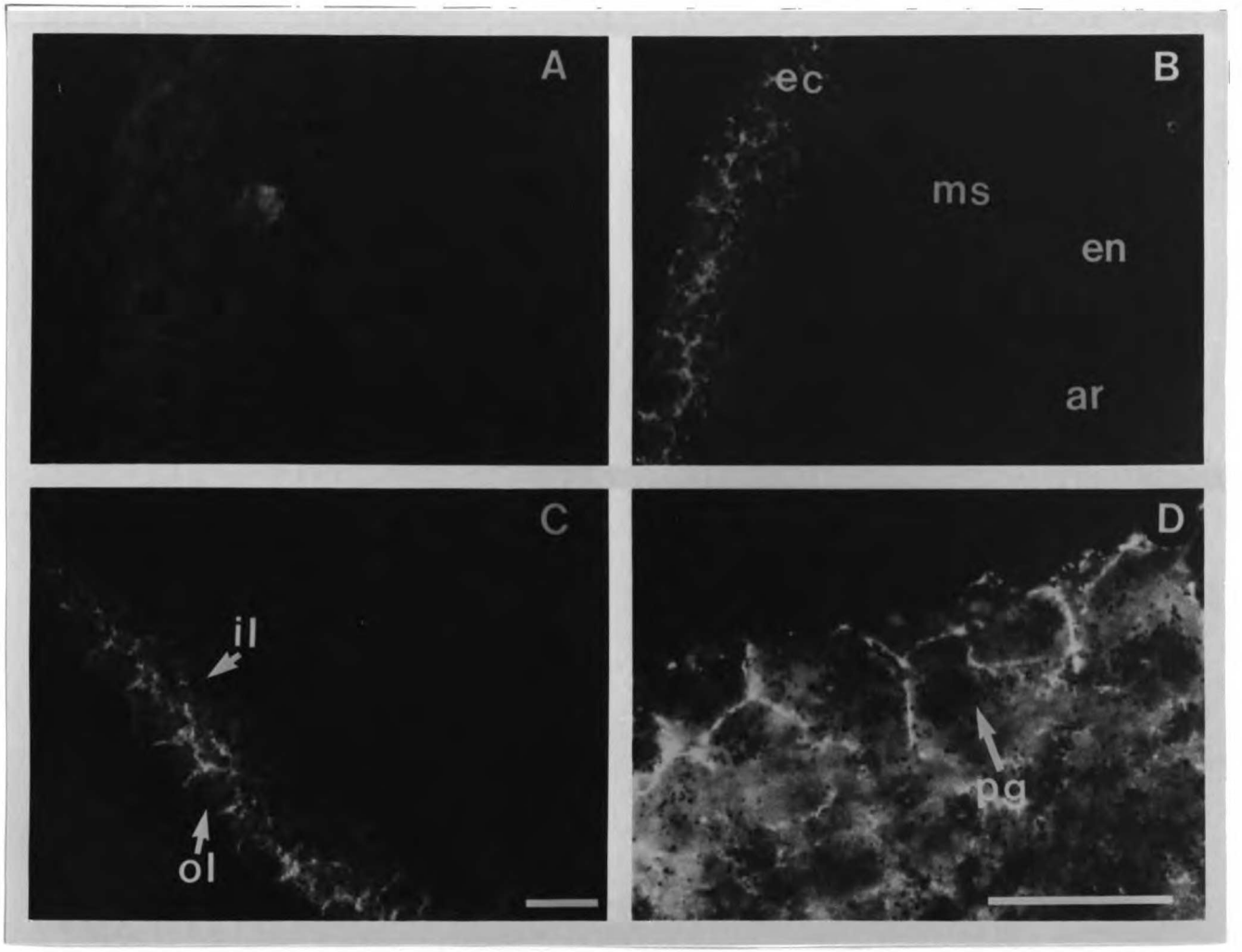
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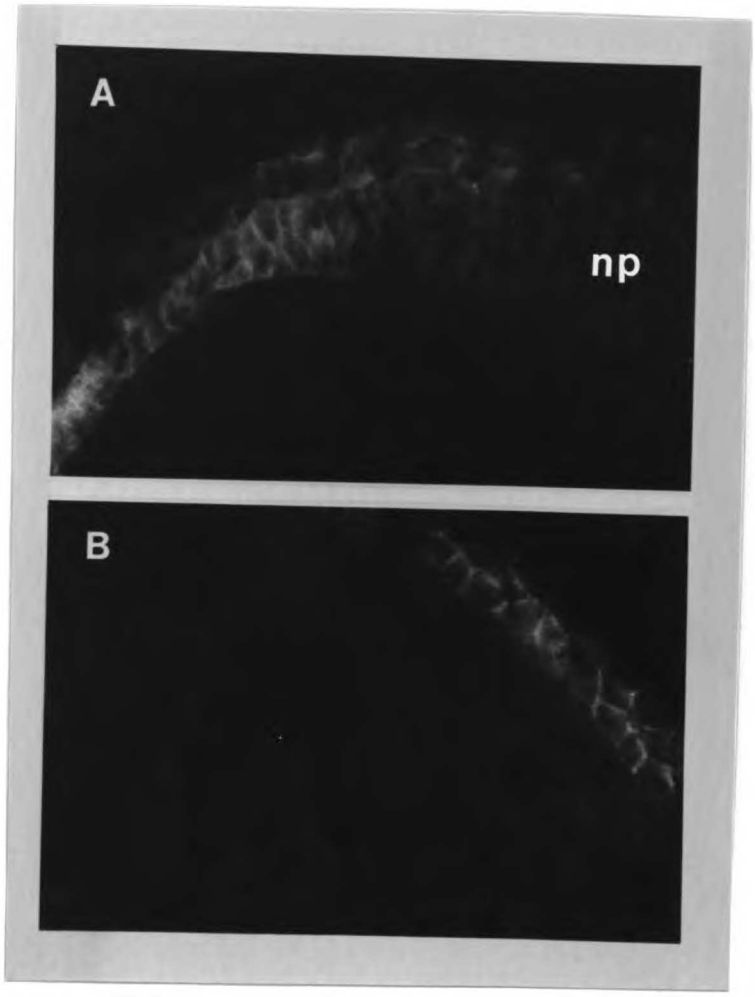
Figure 4. The decrease in E-cadherin expression in the neural fold. Indirect immunofluorescence staining of the stage 17 embryos. Cryostat sections (16 μm) of the embryos prefixed with 3 % TCA were stained with the mixture of monoclonal antibodies 5D3, 8C2, and 19A2, which were detected with Texas-Red conjugated anti-mouse rabbit antibodies. A. Staining of E-cadherin in the neural fold. np (neural plate) B. Staining of E-cadherin in the noninduced ectodermal region. Bar = 40 μm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Chapter 5

Identification of C-cadherin in *Xenopus* Eggs and Dependence of Its Expression in Oocytes on Progesterone Induced Maturation.

Summary

A new cadherin-like protein was identified in oocytes, eggs, and cleavage stage embryos of *Xenopus laevis*. An antiserum raised to a 17 amino acid peptide derived from a highly conserved region in the cytoplasmic domain of all the cadherins which have been sequenced so far, was used as a probe for detecting new cadherin proteins. This anti-peptide antibody recognized with high titer E-cadherin and N-cadherin in *Xenopus*, which were independently identified by monoclonal antibodies specific to these proteins. In extracts of eggs and midblastula stage embryos the anti-peptide antibody recognized specifically an ~120 kD polypeptide that was distinguishable from E-cadherin and N-cadherin by molecular size on SDS gels and by the lack of cross-reactivity of the monoclonal antibodies to E- and N-cadherin. E- and N-cadherin were not even detectable in eggs and midblastula stage embryos. This 120 kD polypeptide has been tentatively named "C-cadherin" due to its presence in the cleavage stages of *Xenopus Laevis*, but a possible relationship to P-cadherin in mouse has not yet been excluded. C-cadherin was synthesized by large, late stage oocytes, and it was induced to accumulate to the levels found in the egg by progesterone induced maturation. It did not accumulate further to any significant extent after fertilization during cleavage up through the midblastula stage. Therefore C-cadherin is a maternally encoded protein that is the major, if not only, cadherin present in the earliest stages of *Xenopus* development, and may play a role in the Ca^{2+}-dependent adhesion and junction formation between cleavage stage blastomeres.

Introduction

All of the cells of the *Xenopus* blastula are linked together by a Ca^{2+} -dependent adhesion mechanism (62, 64, 65). In most cases studied so far, the proteins underlying Ca^{2+} -dependent intercellular adhesion have been found to belong to the cadherin family of adhesion molecules (90). In early mammalian embryos the first cadherin to be expressed is the epithelial cadherin, E-cadherin or uvomorulin (91, 93), and in the chicken (27) it is L-CAM, which is generally believed to be the same as E-cadherin. Because the *Xenopus laevis* blastula forms a tight epithelium, it was expected that E-cadherin would be the first cadherin expressed, and that it would probably be stored in the egg ready to be recruited into the adhesive surface of the cleaving blastomeres. Surprisingly, we were unable to detect *Xenopus* E-cadherin in eggs or early cleaving embryos (11). E-cadherin did not begin to accumulate until the beginning of gastrulation. Thus, the nature of the Ca^{2+} -dependent adhesion system in cleavage embryos remained to be defined.

We wished to examine whether another member of the cadherin family of adhesion molecules, perhaps one that has not yet been identified, might be expressed in the eggs, the early cleavage stage, and the blastula of *Xenopus laevis*. From cDNA clones the amino acid sequences of several different cadherin types in different species are known. The cytoplasmic tail domain has regions of very high homology between different cadherins and between cadherins of

different species (37). An antiserum to one of these conserved regions was generated by B. Gumbiner and P. McCrea with a peptide provided by Dr. L. Reichardt. The anti-peptide antibody was then used as a probe to detect cadherins in *Xenopus* eggs and early embryos.

Result

I. Identification of C-cadherin in the eggs and early embryos of *Xenopus laevis*.

A polyclonal antiserum to the 17 amino acid peptide of the cytoplasmic portion of L-CAM (Fig. 1), whose amino acid sequence is highly conserved between different cadherins and cadherins of different species (37), was generated by P. McMrea and B. Gumbiner using a peptide provided by Dr. L. Reichardt. It was shown to cross-react with *Xenopus* E-cadherin in A6 cells by immunoblotting and by immunoprecipitation (P. McCrea and B. Gumbiner, unpublished observation). The anti-peptide antibody recognized a ~ 120 kD glycoprotein in the ConA enriched glycoprotein fraction of *Xenopus* egg extract, which was not detected by a preimmunine serum. After preincubation with excess peptide, the anti-peptide antibody no longer recognized the 120 kD polypeptide in the egg extracts (R. Sehgal and B. Gumbiner, unpublished observation).

The 120 kD putative cadherin expressed in the *Xenopus* eggs was distinct from E-cadherin and N-cadherin (Fig. 2). The anti-peptide antibody recognized the 120 kD glycoprotein in extracts of *Xenopus* eggs (arrow head at lane a) and embryos at the midblastula transition (MBT) stage (~ 7 hr after fertilization) (lane d). In addition to the 120 kD polypeptide, the anti-peptide antibody also recognized two other bands migrating at ~140 kD and ~130 kD which became more intense in the

MBT stage embryos. However, none of these polypeptides were recognized by mAbs to *Xenopus* E-cadherin (lane c, f) or by mAb NCD-2 raised against chicken N-cadherin (lane b, e) which cross-reacts with *Xenopus* N-cadherin (lane d in Fig. 4 and M. Takeichi, personal communication). Therefore the 120 kD polypeptide present in the eggs and early embryos is likely to be another member of cadherin gene family. I could not rule out, however, the possibility that the 120 kD polypeptide in eggs is P-cadherin (Fig. 4). We named the 120 kD polypeptide as cleavage specific cadherin (C-cadherin) because it was suggested by B. Gumbiner that this molecule may play a role in the cell-cell adhesion and the assembly of intercellular junctions of cleaving embyros at the early stage even before E-cadherin and N-cadherin are expressed.

The level of C-cadherin polypeptide expression increased gradually during the early stage of embryonic development. Until the midblastula transition (MBT), embryonic gene transcription does not occur (53). However some proteins are synthesized from stored mRNAs during this period. To determine whether C-cadherin molecules are synthesized during the early cleavage stages, an equal number of eggs and embryos were extracted with NP-40-containing buffer at 2 hr intervals up until the MBT stage and immunoblotted with the anti-peptide antibody (Fig. 3). During this time period, more 120 kD polypeptides were observed to accumulate even though the immunoblotting assay is not a sensitive method for quantitative analysis (lane a-e). Two minor bands migrating at higher molecular mass (~140 kD and ~130 kD) became more prominent as embryonic

development proceeded up to the MBT stage. Since the anti-peptide antibody could react with all cadherins with a common cytoplasmic domain, I was unable to examine whether these polypeptides are newly synthesized precursors of the 120 kD polypeptide or distinct cadherins yet to be identified. However, the presence of C-cadherin in the eggs at high level and new synthesis of C-cadherin during the early stages of the embryonic development indirectly suggest that C-cadherin may be an essential molecule for the development of the early cleaving embryos.

All three polypeptides detected by the anti-peptide antibody have the peptide common to all the known cadherins because they were undetectable after competition of the antibody with excess peptides (Fig. 3, lane f-j). Therefore the 120 kD polypeptide is C-cadherin, and 130 kD and 140 kD polypeptide may be precursors to it or to other cadherins sharing the highly conserved cytoplasmic domain.

II. Expression of C-cadherin in other tissues

C-cadherin seems to be expressed not only in eggs and embryos but also in some adult tissues of *Xenopus laevis* (Fig. 4). All the known cadherins tend to be expressed in a tissue specific manner even though there exists some overlap between tissues; E-cadherin in the epithelial tissues, N-cadherin in the neural tissues, and P-cadherin in the mesothelium, skin and placenta. Therefore A6 cells (lane a, b), brain tissues (lane c, d), peritoneum (lane e, f), and skin (lane g, h) were used as tissues that normally express E-, N-, and P-cadherin respectively. The monoclonal antibody NCD-2 recognized a 140 kD N-cadherin

polypeptide in *Xenopus* brain (lane d). The anti-peptide antibody recognized a 120 kD polypeptide in A6 cells (lane b), the brain (lane c), and peritoneum (lane e) in addition to the 140 kD E-cadherin polypeptide (lane b) and the N-cadherin polypeptide (lane c) by an immunoblotting assay. The 120 kD polypeptide in A6 cells, brain, and peritoneum were not recognized by monoclonal antibodies to *Xenopus* E-cadherin (lane a, g) or monoclonal antibodies to chicken N-cadherin (lane d). The 120 kD polypeptide in A6 cells is probably not a degradation product of E-cadherin because none of the degradation products of E-cadherin detected by anti-E-cadherin antibodies were recognized by the anti-peptide antibody, probably as a result of the loss of the cytoplasmic domain during degradation (compare lane a and b). The 120 kD polypeptides present in the A6 cells, brain, and peritoneum, which are distinct from E-cadherin and N-cadherin, may be the same as C-cadherin expressed in the eggs and early cleaving embryos. However I was unable to exclude the possibility that they are other members of the cadherin family yet to be identified. This experiment will be possible after a specific probe to C-cadherin is made.

The anti-peptide antibody recognized a 140 kD polypeptide in the extracts of peritoneum (lane f) and skin (lane h), probably E-cadherin because E-cadherin antibodies also detected a 140 kD polypeptide (lane e and g). I was unable to rule out the possibility that C-cadherin is the *Xenopus* equivalent of P-cadherin in this study because a specific probe to detect P-cadherin in *Xenopus laevis* is not available and the tissues which express P-cadherin at the highest level such as placenta and hair cells (67) do not exist in the amphibian.

III. Expression of C-cadherin polypeptide in oocytes is dependent on progesterone induced maturation.

The maturation of *Xenopus* oocytes by progesterone increases the level of the C-cadherin polypeptide in the oocytes up to that present in the eggs. Maturation of *Xenopus* oocytes is accompanied by many biochemical changes (2, 42, 57). To determine when the C-cadherin polypeptide is synthesized during the development of oocytes, the levels of the C-cadherin polypeptide were examined in the unmatured oocytes and in progesterone matured oocytes. Premeiotic *Xenopus* oocytes at stage 6 were induced to mature *in vitro* with 5 μg progesterone/ml for 5 hr (5). The completion of maturation was judged by the breakdown of the germinal vesicle (GVBD). After GVBD, the oocytes were incubated in MBSH for 20 hr to allow a greater accumulation of proteins synthesized during the maturation process. In the stage 6 oocytes incubated overnight *in vitro* , C-cadherin was present at low but detectable levels (Fig. 5 A, lane a). However, when the same batch of oocytes was induced to mature with 5 hr progesterone treatment and then incubated overnight *in vitro*, the C-cadherin polypeptide accumulated to the same levels as it is found in naturally layed eggs (Fig. 5 A, lanes b and c). The biosynthesis of the C-cadherin polypeptide by oocytes was confirmed by immunoprecipitating a denatured extract of oocytes that had been labeled with ^{35}S-methionine for 5 hr (Fig. 5 B). A biosynthetically labeled 120 kD polypeptide was immunoprecipitated by the anti-peptide antibody (lanes a and c), but not in the presence of excess cold cadherin-derived peptide (lane b). These results show that C-cadherin is

synthesized by late stage oocytes, and that its accumulation to the levels found in the egg is dependent on maturation induced by progesterone.

Progesterone did not seem to substantially increase the rate of C-cadherin synthesis during the first 5 hr of treatment (compare lanes a and c in Fig. 5 B). The accumulation of C-cadherin during *in vitro* overnight incubation could result either from a gradual increase in translating mRNA after the 5 hr maturation process or from a decrease in the rate of turnover of the protein.

Discussion

The anti-peptide serum used in this study seems to be a good probe for the detection of a wide variety of cadherins in different species. Although raised to a peptide sequence derived from the known chicken and mouse cadherins (29), it specifically recognized cadherins in tissues of *Xenopus laevis*. The antiserum bound to E-cadherin in the extracts of cultured A6 cells, skin and peritoneum, and to N-cadherin in brain extracts. The specificity of the antiserum for the cadherin sequence was readily demonstrated by the inhibition of its binding to cadherins by an excess of the peptide against which it was raised. These findings suggest that the anti-peptide antibody is capable of recognizing many, if not all, members of the cadherin family of proteins in tissues of *Xenopus laevis*. Similar conclusions have been reached from observations by Lilien and Reichardt examining independently derived antisera to the same peptide (unpublished observation). Therefore, this antibody might be expected to be a suitable reagent for the identification of new member of the cadherin family of proteins in *Xenopus laevis* .

We have used this antibody to detect a cadherin-like protein of unknown identity in oocytes, eggs, and cleavage stage embryos. Unfortunately, it has not yet been possible to use other established criteria for identifying cadherins, such as the characteristic Ca^{2+} - dependent change in molecular conformation. The characteristic protection of the ectoplasmic domain from trypsin degradation by calcium ions (34, 43) could not be demonstrated, because the antibody

only recognizes the protein after denaturation and presumably only recognizes the cytoplasmic domain, for which the sensivity to trypsin and /or calcium ions is not known. Specific antibodies that bind to the ectoplasmic domain will be required for such experiments. However, because the antibody used to detect this protein was raised against a specific cadherin consensus sequence and because it recognized E- and N-cadherin in *Xenopus* tissues, we believe it is very likely that the egg protein is a maternally encoded cadherin-like protein. Eventually the amino acid sequence of C-cadherin determined from its cDNA clone will reveal its overall relatedness to the other cadherin.

The presence of this cadherin in eggs and cleaving embryos could account for the Ca^{2+} -dependent adhesion system that has been shown to be present in early *Xenopus* blastomeres (62, 64, 65). We have tentatively named it "C-cadherin" because of its occurance in cleavage stage embryos. Unfortunately its functional activity cannot be investigated with the usual adhesion assays, because the anti-peptide antibody used in this study recognizes the cytoplasmic tail rather than the extracellualr domain, and only under denaturing conditions. Again such experiments will require new antibodies raised to the extracelluar portion of the protein. Nevertheless, the finding that C-cadherin is expressed by late stage oocytes in response to progesterone-induced maturation is highly suggestive that this protein plays a role in the early stages of development before the onset of zygotic gene transcription.

The increase in C-cadherin expression during meiotic maturation is most likely an indirect post-transcriptional response to progesterone.

Progesterone-induced maturation is a complex process that begins with the activation of a cell surface receptor and involves many cellular events which lead to germinal vesicle breakdown (GVBD) (57, 79). The increased expression of oocyte proteins during maturation results from translational regulation of stored maternal mRNAs by a mechanism involving mRNA recruitment onto polysomes (77). The early induction of a small number of proteins, including the c-mos proto-oncogene, is required for meiotic maturation and GVBD (80). Quantitatively, however, the greatest hormone-induced increase in protein synthesis occurs after GVBD (57, 77). These "late" proteins, including for example the histones, are believed to be destined to function in later stages of development. C-cadherin expression is similar to the late induced proteins, because it accumulates gradually after progesterone-induced maturation and its rate of biosynthesis does not increase significantly during the initial 5 hr period of progesterone treatment. Although we have not ruled out regulation of C-cadherin expression by a decrease in its rate of turnover, the similarity of its pattern of expression to that of the known late-induced proteins suggests that its expression is regulated at the level of translation.

The relationship of C-cadherin to the other well defined cadherins is not completely clear, in part because not all cadherin antibodies cross-react across species. In other species, E-cadherin or L-CAM has been found to be expressed at the earliest satges of embryonic development (91, 93), but we were unable to detect *Xenopus* E-cadherin until the time of gastrulation (11). In the present study we show that C-cadherin is distinct from E-cadherin and N-cadherin, by both criteria of

molecular size and the lack of antibody cross-reactivity. It remains possible that C-cadherin is the *Xenopus* version of P-cadherin, since it has not yet been possible to identify P-cadherin in *Xenopus*. It is also conceivable that C-cadherin is the protein in *Xenopus laevis* which is recognized by antibodies to chicken L-CAM, rather than E-cadherin, as proposed previously (55). Antibodies to chicken L-CAM seem to detect a polypeptide of ~120 kD in *Xenopus* skin and perhaps weak immunohistological reactivity in blastula stage embryos (55). To determine the definitive relationships between C-cadherin and other known cadherins, the generation of additional antibodies specific to *Xenopus* C-cadherin and/or the cloning of several of the *Xenopus* cadherin cDNAs will be required.

C-cadherin may be present with other cadherins in tissues other than the egg or blastula. In the A6 kidney cell line, brain and perhaps mesothelial tissues the anti-cytoplasmic tail peptide antibody specifically recognized polypeptides of ~120 kD that were not recognized by monoclonal antibodies to E-cadherin or N-cadherin. The similarity of their molecular sizes on SDS gels to C-cadherin raises the possility that they are all the same protein. Such a conclusion is premature, however, because the anti-peptide antibody has a broad specificity for most, if not all, cadherins.

In some experiments minor bands of a molecular weight slightly greater than the 120 kD C-cadherin were resolved from the major C-cadherin polypeptide. These might represent biosynthetic precursors or post-translational modifications of C-cadherin. Larger biosynthetic precursors having an N-terminal propeptide have been observed for

all of the known cadherins (37). Again, the broad specificity of the anti-peptide antibody makes it difficult to know the relationship between all of these polypeptides. It is clear, however, that these bands are not recognized by monoclonal antibodies to E-and N-cadherin. Specific probes for C-cadherin and pulse-chase metabolic labeling experiments will enable us to examine whether they are metabolically related to C-cadherin, or whether they represent yet additional cadherin species in *Xenopus*.

We propose that C-cadherin is present in the *Xenopus* egg for the purpose of mediating Ca^{2+}-dependent adhesion and/or epithelial junction formation between early cleavage stage blastomeres. Our findings suggest that C-cadherin is synthesized by the oocyte during its maturation to a fertilizable egg and is stored in the egg for recruitment to the cell surface during early development. Although turnover and new synthesis of C-cadherin during early cleavage stages cannot be excluded from our data, C-cadherin levels do not increase a great deal until the midblastula stage. Future experiments will investigate the structure and function of C-cadherin and the mechanism by which it is recruited into and aids in the biogenesis and regulation of adhesive junctions during embryonic development.

Figure 1. Peptide derived from conserved cytoplasmic domain of cadherins. The peptide was derived from amino acid residues 665-681 derived from the chicken L-CAM cDNA sequence (29).

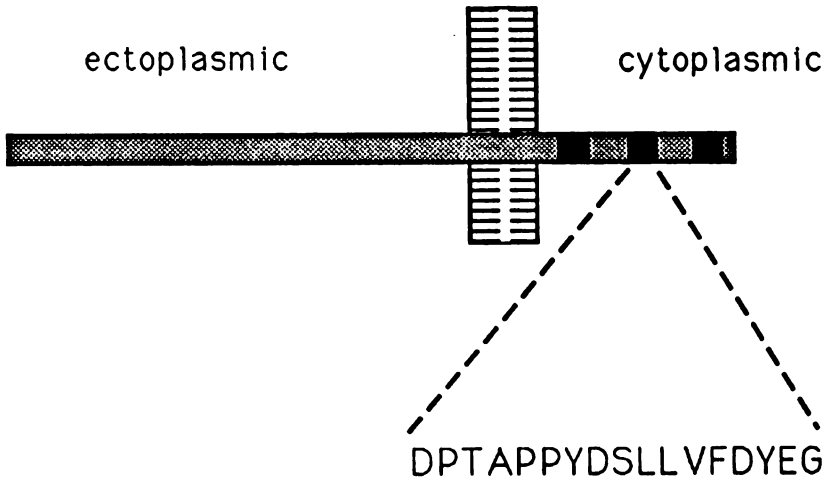

ectoplasmic
cytoplasmic
DPTAPPYDSLLVFDYEG

Figure 2. Identification of a novel C-cadherin in the *Xenopus* eggs and embryos. Extracts of ~ 200 *Xenopus* eggs (lane a-c) and embryos at midblastula stage (lane d-f) were immunoblotted with the anti-peptide antibody against cadherin-derived peptide at a dilution of 1 to 500 (lane a, d), a monoclonal antibody against chicken N-cadherin (NCD-2) (lane b, e), and a mixture of monoclonal antibodies (5D3, 8C2, and 19A2) against *Xenopus* E-cadherin (lane c, f). Numbers at left are molecular weight markers in kilodaltons.

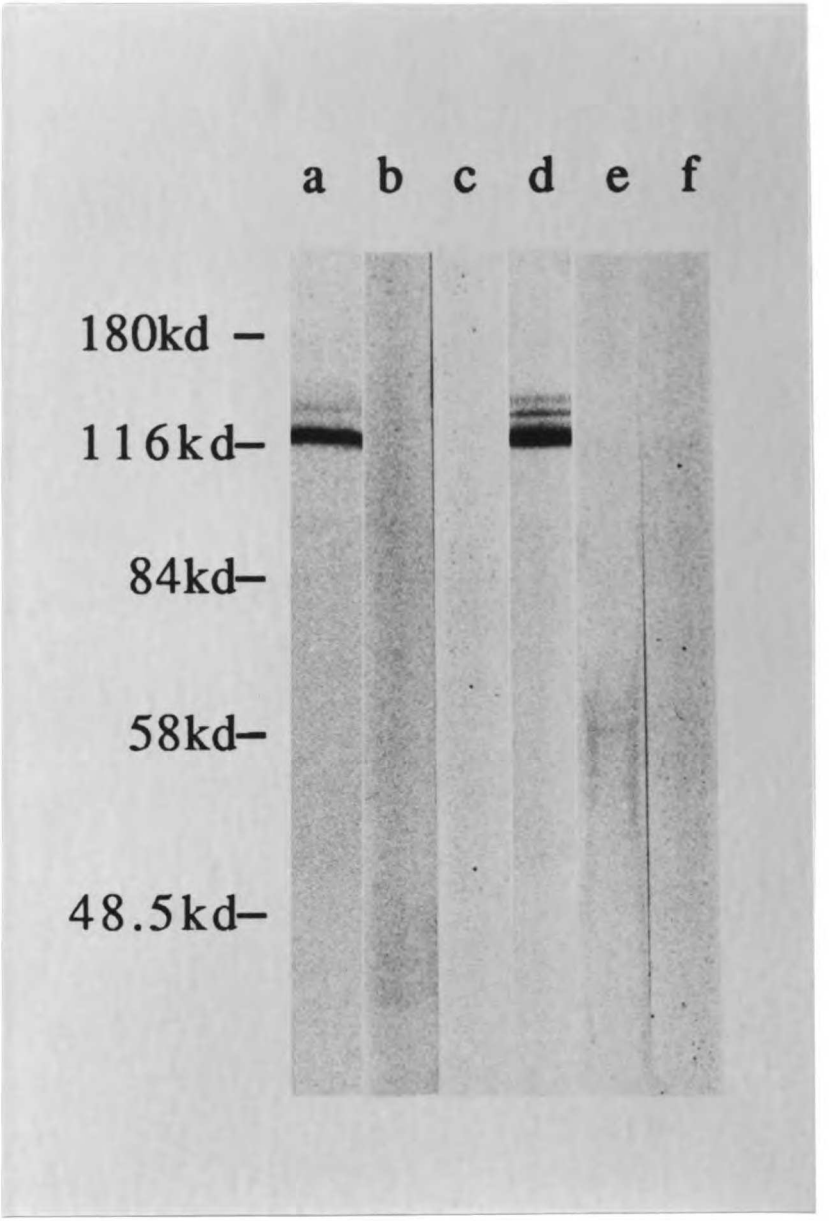

a b c d e f
180kd –
116kd–
84kd–
58kd–
48.5kd–

Figure 3. C-cadherin expression during early cleavage stage development of *Xenopus laevis*. Extracts of ~200 eggs (lane a, f) and embryos at 2 hr (lane b, g), 4 hr (lane c, h), 6 hr (lane d, i), and 7 hr (lane e, j) after fertilization were immunoblotted with 1 to 500 dilution of the anti- peptide antibody (lane a-e) and the same serum after preincubation with 30 μg peptide (lane f-j). Numbers at left are molecular weight markers in kilodaltons.

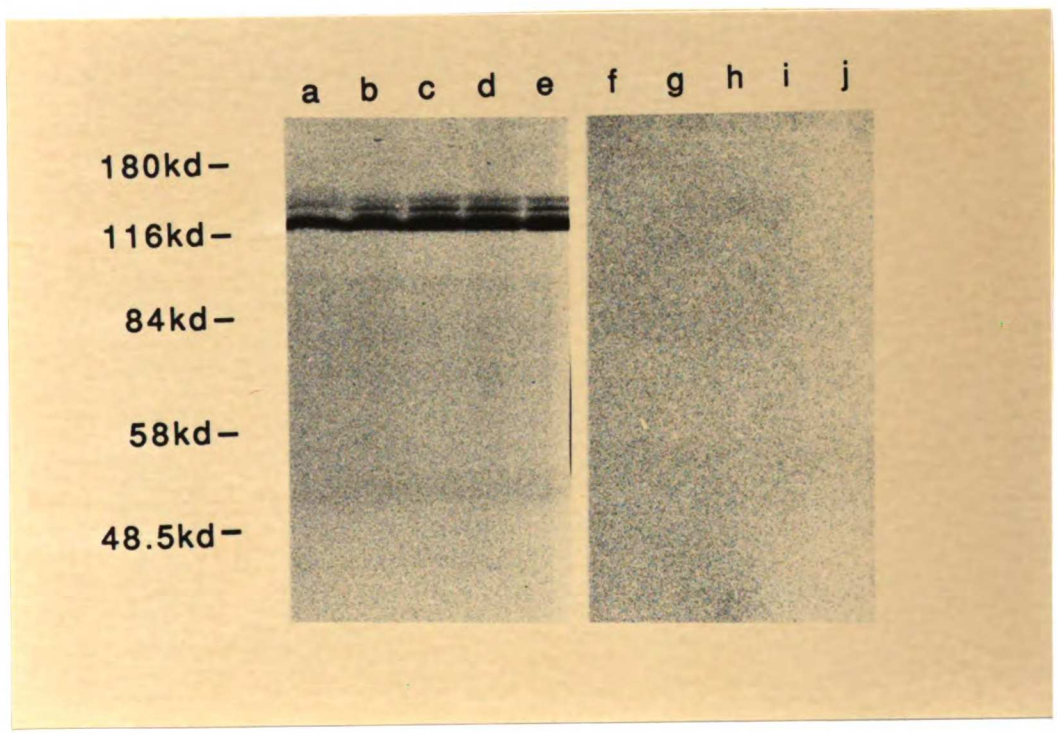

a b c d e f g h i j
180kd–
116kd–
84kd–
58kd–
48.5kd–

Figure 4. Anti-peptide antibody recognizes cadherins in other tissues. Extracts of A6 cells (a, b), brain (lane c, d), peritoneum (lane e, f), and skin (lane g, h) were immunoblotted with 1 to 500 dilution of the anti-peptide antibody (lane b, c, f, h), with monoclonal antibody NCD-2 against chicken N-cadherin (lane d), and with the hybridoma supernatants against *Xenopus* E-cadherin (lane a, e, g). Numbers at left are molecular weight markers in kilodaltons.

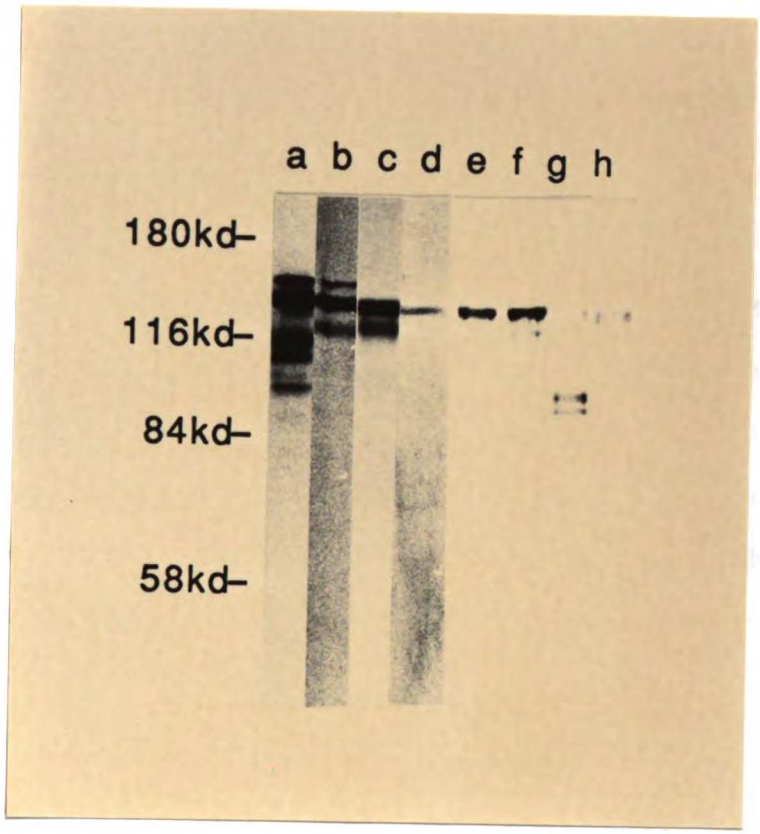
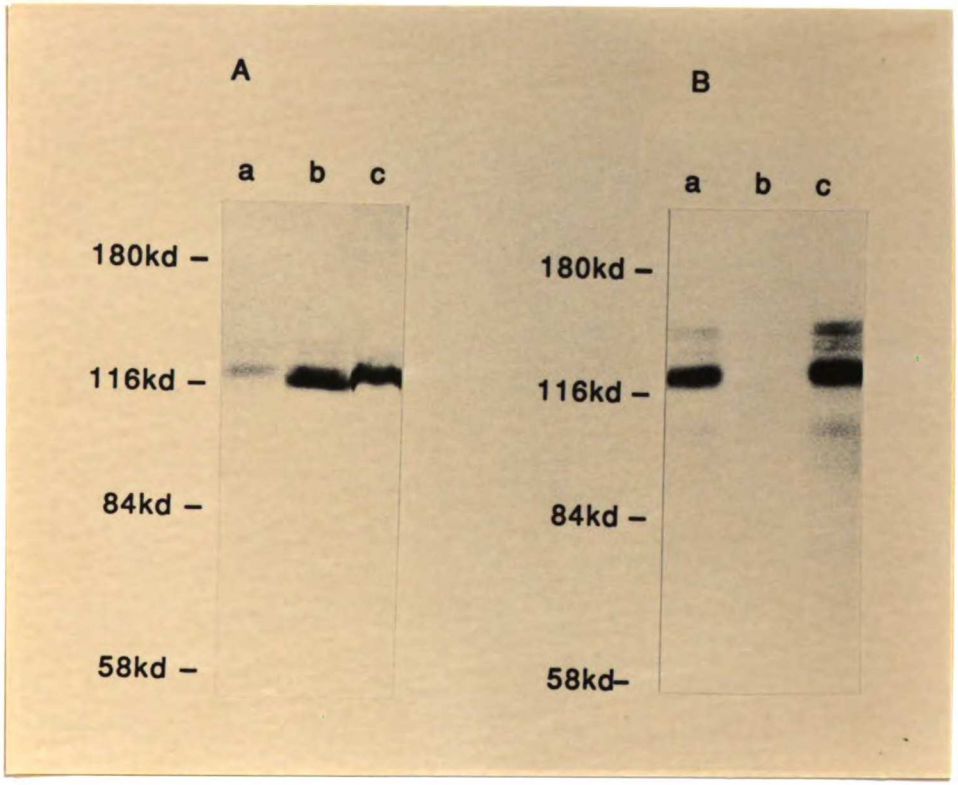

a b c d e f g h
180kd–
116kd–
84kd–
58kd–

Figure 5. C-cadherin expression in oocytes and dependence on progesterone induced maturation. A. Levels of C-cadherin expression in progesterone treated and untreated oocytes at 20 hr maturation *in vitro*. Oocytes were incubated for 5 hr with (lane b) or without (lane a) 5μg progesterone and subsequently incubated in MBSH free of progesterone for 20 hr. ~ 200 oocytes from each were extracted and immunoblotted with a 1 to 500 dilution of the anti-peptide antibody. For comparison an extracts of 200 eggs was also immunoblotted with the same serum (lane c). B. Biosynthetic labeling of C-cadherin polypeptides. Oocytes were labelled with 1 mCi of ^{35}S-methione for 5 hr with (lane c) or without (lane a) progesterone. Extracts were SDS- and heat-denatured, and immunoprecipitated with the anti-peptide antibody (lane a, c) or with the anti-peptide antibody after preincubation with 30 μg cadherin-derived peptide (lane b).


A
a
b
c
180kd –
116kd –
84kd –
58kd –
B
a
b
c
180kd –
116kd –
84kd –
58kd–

Chapter 6

Experimental Procedures

1. Cell culture and resistance recovery assay

The A6 *Xenopus* kidney epithelial cell line was obtained from the American Type Culture Collection (ATCC) and subcloned by limiting dilution. The subclone A6.2 was used throughout these experiments. The cells were grown at 28°C in a humidified atmosphere of 1 % CO_2 in air. The growth medium was 85 % DMEM H16 containing 0.85 g/L glucose and 0.74 g/L $NaHCO_3$, supplemented with 5 % SCS (supplemented calf serum), 10 mM Hepes pH 7.4, and 2 mM glutamine. For routine passage, cells were dissociated into single cells by treatment with 0.05 % trypsin/0.02 % EDTA and seeded at a 1: 5 ratio into 75 cm^2 flasks. They became confluent at 4 days, and the growth medium was changed once at 3 days after seeding.

For the resistance recovery assay, cells were plated onto 12 mm Millicell HA filter chambers (Millipore Co., Bedford, MA) at a density of 2×10^5 cells/filter and grown for 7 days in the growth medium supplemented with 10^{-7} M dexamethasone. Filters that had a transepithelial resistance greater than 4,000 ohm x cm^2 were chosen for this assay. The assay was similar to that described (34) with modification. Cells on filters were washed 3 times in 85 % phosphate buffered saline (PBS; 0.15 M NaCl, 15 mM Na_2HPO_4, pH 7.5). To open intercellular junctions, filters were incubated in 85 % SMEM (Gibco Lab., Chagrin Falls, OH) supplemented with 1 % dialyzed FCS (34) at 28°C for 6.5 min. Filters were then transfered into humidified wells of a 24 well culture plate, and incubated with 100 µl of a 1:50 dilution of mAb ascites fluid made up in recovery medium (85 % SMEM

supplemented with 1 % dialized FCS and 1 mM $CaCl_2$) for 40 min on ice while rocking gently. Filters were then transfered to recovery medium at 28°C, and transepithelial resistance was measured at indicated times.

2. Tryptic digestion of whole cells.

Confluent A6 cells were washed 3 times with 85 % PBS^+ (PBS^+; 0.15 M NaCl, 15 mM Na_2HPO4 pH 7.5, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$), and scraped into either solution A (10 mM Hepes pH 7.4, 150 mM NaCl, 2 mM $CaCl_2$) or solution B (10 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM EDTA) on ice at the density of 5×10^6 cells/ml. Cells were incubated with 0.2 mg/ml trypsin (Sigma Chemicals, St. Louis, MO), shaken gently for 30 min at 37°C, and the digestion terminated by the addition of 0.3 mg/ml soybean trypsin inhibitor (Sigma Chemicals, St. Louis, MO), 1 mM PMSF (Sigma Chemicals, St. Louis, MO), and 1 mM iodoacetamide (IAA). Cells were removed by ultracentrifugation at 30,000 rpm for 1 hr in a Ti 80 rotor (Beckman Instruments Inc. Palo Alto, CA). To concentrate glycoproteins in the supernatant for some experiments, the supernatant from 5×10^6 cells was adjusted to 0.5 % Triton X-100, mixed with 50 µl of 1:1 slurry of Con A-sepharose-4B (Sigma Chemicals, St. Louis, MO), and incubated rotating for 1 hr at 4°C. Sepharose beads were pelleted at 1,000 rpm for 5 min in a microcentrifuge, washed 3 times with immunoprecipitation buffer (1 % Triton X-100, 0.5 % Na deoxycholate, 0.2 % SDS, 0.15 M NaCl, 20 mM Hepes pH 7.4), once with high salt buffer (0.5 M NaCl, 10 mM Tris pH 7.4), and once with 10 mM Tris buffer pH 7.4.

3. Extraction of A6 cells with Triton X-114 and tryptic digestion of aqueous phase of Triton X-114 extract.

Confluent A6 cells were washed 3 times with 85 % PBS^+ and extracted on ice with 1 % Triton X-114 in solution A supplemented with a protease inhibitor cocktail (1 mM PMSF, 1 mM IAA, 1 mM benzamidine, 10 μg/ml of aprotinin, pepstatin, leupeptin and antipain respectively). The phase separation was done as described (7). The Triton X-114 insoluble pellet was dissolved in 2 % SDS extraction buffer (2 % SDS, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM PMSF, 1 mM IAA). The same relative fractions of pellet, detergent phase, and aqueous phase were directly mixed with SDS sample buffer for SDS-PAGE. To load more proteins on SDS-PAGE for immunoblotting with polyclonal serum to canine E-cadherin, glycoproteins in the aqueous phase were concentrated as described above.

For tryptic digestion of the aqueous phase of Triton X-114 extracts of A6 cells, cells were extracted with 1 % Triton X-114 in the solution A without protease inhibitors. After phase separation, the aqueous phase was divided into two tubes. For the tryptic digestion in the absence of Ca^{2+}, one tube was treated with 3 mM EDTA for 5 min on ice to chelate Ca^{2+}. Trypsin digestion was done as described above.

4. Extraction of tissues from adult *Xenopus laevis*.

The liver was removed from adult *Xenopus laevis*, cut into small pieces with scissors, rinsed twice with ice cold 85 % PBS^+, and then homogenized in 85 % PBS^+ supplemented with protease inhibitors (10

to 1 vol to tissue) in a waring blender at low speed for 1 min at 4°C (all following procedures undertaken at 4°C). The homogenate was filtered through gauze to remove remaining connective tissue and centrifuged at 7,000 rpm for 10 min in a Beckman JA 20 rotor. The pellets were resuspended in immunoprecipitation buffer supplemented with protease inhibitors (5 to 1 vol to tissue) and homogenized with 5 strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, NJ). Extracts were centrifuged at 12,000 rpm for 30 min in a JA 20 rotor and the supernant was ultracentrifuged at 30,000 rpm for 1 hr in a Beckman Ti 70 rotor to remove all the insoluble material. Lung, skin, peritoneum, and brain were directly homogenized in immunoprecipitation buffer with a Dounce homogenizer. Protein concentrations were measured by the amido schwartz method (82). Equal protein amounts of each extracts were concentrated by binding to Con A-sepharose-4B and SDS-PAGE was carried out.

5. *Xenopus* eggs and embryos

Production of eggs, fertilization and removal of the jelly coat were done as described (62). Fully mature female frogs were subcutaneously injected with 100 U pregnant mare serum gonadotropin (Gestyl; Diosynth co., Chicago, IL) to sensitize oocytes at 4-7 days prior to use. Sensitized frogs were induced to lay eggs by injection with 700 IU human chorionic gonadotropin (Sigma Chemicals, St.Louis, MO), 8-12 hr before use at 23°C or 16 hr before use at 16-18°C. To reduce spontaneous activation of layed eggs, injected frogs were placed in 1 x MMR (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes pH 7.8, 0.1 mM EDTA pH 7.8). Eggs layed in 1 x MMR were used

for extraction. For fertilizing small numbers of eggs, eggs were directly squeezed from the frogs into 60 mm petri dish containing 10 ml 1 x MMR. After decanting most of the 1 x MMR, eggs were rubbed with a testis that has been lightly teased open at one end with sharp forcep just before use (The highest efficiency of fertilization was obtained when the eggs covered the surface of a petri dish as a single layer.). After 2-5 min (5 min for 1 week old testis), the sperm was activated by adding 10 ml 0.1 x MMR to the eggs and sperms (This is t = 0 of fertilization). To obtain large numbers of embryos, the eggs layed in 1 x MMR were fertilized. Fertilization efficiency under these conditions was ~ 80 %. Unfertilized eggs were sorted out at the 4 cell stage (~2.5 hr after fertilization). At 20 min or more after fertilization, fertilized eggs can be distinguished from unfertilized eggs. Fertilized eggs rotate and sit straight upright (animal side up, vegetal side down). The dark pigment of the animal side contracts, and become smaller and darker. The fertilized eggs become more turgid and tend to bounce away from forceps which contact them. At this time, embryos can be dejellied with 10 ml of 2 % cysteine-HCl in H_2O (Sigma Chemicals, St. Louis, MO) pH 7.8 with NaOH, while swirling occasionally until the jelly coats are dissolved (~5 min). The fertilized eggs were washed several times with 0.1 x MMR to remove excess cysteine. The first cleavage begins at 1.5-2 hr after fertilization at 20-23°C and the time required for each subsequent cleavage is about 30 min at 20°C up until the midblastula transition (MBT; 6-7 hr after fertilization). For embryos older than the MBT stage, embryos were kept at 16-19°C. When embryos were moved from one dish to another, a polished pasteur pipet was utilized so as not to damage the embryos.

6. Extraction of eggs and embryos.

Dejellied eggs or embryos were extracted with an equal volume of 1 % NP-40 in solution A supplemented with protease inhibitors. This procedure avoided disruption of the yolk platelets. The yolk platelets and other insoluble material were removed by centrifugation at 10,000 rpm for 30 min in a microcentrifuge. Yellow lipid droplets on top were carefully removed and the supernatant was incubated with Con A-sepharose-4B or mAbs coupled to sepharose-4B. In some experiments, eggs or embryos were extracted with immunoprecipitation buffer, which tends to solubilize yolk proteins as well.

7. SDS-PAGE and immunoblotting.

SDS-PAGE and immunoblotting were performed as described (34, 36). Proteins were separated on 8 % polyacrylamide/0.21 % bis-acrylamide gel under denaturing condition. Prestained mol wt markers (Sigma Chemicals, St. Louis, MO) were used for the molecular mass estimation of proteins.

Proteins on the gel were electrophoretically transfered to nitrocellulose overnight at a constant current of 100 mA. After washing the nitrocellulose paper with 20 % methanol for 5 min, it was stained and fixed with 0.2 % Ponceau-S (Eastman Kodak Co, Rochester, NY) in 3 % TCA for 10 min. After washing away excess dye, the nitrocellulose was cut into strips and each strip was incubated with blocking buffer (5 % nonfat dry milk dissolved in PBS, 0.2 % Triton X-100 and filtered through Whatman filter paper) for 1 hr. Polyclonal

serum diluted with blocking buffer or hybridoma culture supernatant were used as first antibodies. Alkaline phophatase-conjugated goat anti-rabbit IgG and anti-mouse IgG (Bio-Rad Lab., Richmond, CA), and alkaline phosphate conjugated rabbit anti-rat IgG (Miles Lab. Inc., Elkhart, IN) were used as second antibodies to detect polyclonal, mouse mAbs, and rat mAbs respectively. Each antibody treatment proceeded for 1 hr at room temperature and nitrocellulose strip washings occurred over 1 hr with 3 changes of blocking buffer. In some experiment, to increase the sensitivity for detecting proteins in egg and embryo extracts, a two antibody detection method was used. The mouse primary antibodies were detected by firstly incubating with rabbit anti-mouse IgG, and subsequently with alkaline phosphatase-conjugated goat anti-rabbit IgG. NBT and BCIP (Bio-Rad Lab., Richmond, CA) were used as color developing reagents.

In one experiment, to confirm that the protein band in Coomassie blue stained gels was the E-cad100 polypeptide, proteins from a fixed and Coomassie blue stained gel were electrophoretically transfered to nitrocellulose paper after extensive destaining with 50 % methanol, 7 % acetic acid and equilibrating with running buffer (50 mM Tris base, 0.384 M glycine, 0.1 % SDS) as described (74)

The microaffinity purification of antibodies bound to proteins immobilized on nitrocellulose was done as described with modifications (8). The extracellular tryptic fragment of canine E-cadherin, purified as described (36), was run on preparative SDS-PAGE, and transfered to nitrocellulose paper. The protein band was located by staining with Ponceau-S and cut out for antibody purification.

Antibodies bound to canine E-cadherin immobilized on a nitrocellulose strip were eluted off with 0.2 M glycine pH 2.0 for 2 min at room temperature and neutralized with 1 M Tris base right away.

8. Preparation of antigen and generation of monoclonal antibodies

Large amounts of the E-cad100 polypeptide were prepared from trypsin digests of A6 cells by scaling up the procedure described above. The supernatant was dialyzed with column buffer (10 mM Hepes pH 7.4, 2 mM $CaCl_2$, 50 mM NaCl), loaded on to a pre-equilibrated DE-52 column (1.6 cm x 5.0 cm), and eluted with a linear gradient of NaCl (0.05 M to 0.5 M). The fractions were analyzed by immunoblotting with the polyclonal serum to canine E-cadherin. The E-cad-100 containing fractions eluted between 0.1 M and 0.2 M NaCl were pooled, bound to Con A-sepharose-4B (~ 80 µg Con A / 2 x 10^7 cells / 75 cm^2 flask), and washed as described above. E-cad100 was further purified by preparative SDS-PAGE (8 % acrylamide/0.21 % bis-acrylamide gel). The gel was stained lightly, destained, the E-cad100 band cut out, and washed with distilled water for 2 hr with frequent changes. After this step, gel slices may be stored at - 20°C. For immunization, gel slices containing approximately 5 µg of E-cad100 were finely minced between two plastic plates of a petri dish, suspended in 0.3 - 0.5 ml PBS, and emulsified with the same volume of complete Freund's adjuvant (Calbiochem Co., La Jolla, CA) for the first injection, and incomplete Freund's adjuvant for the second injection. Balb/C mice were injected first subcutaneously and boosted i.p. after 2 weeks. At the 3rd week, the serum titer was checked by immunoblotting. A mouse whose titer was 1:1,000, was chosen and boosted finally i.p. with approximately 20 µg of

E-cad100 in PBS. The fusion and culture of hybridomas was done as described (10). SP-2/O myeloma cells (T. De Franco Lab., UCSF) were fused with immunized spleen cells using 50 % polyethyleneglycol (Boehringer Mannheim Biochemicals, Indianapolis, IN). Macrophages obtained from peritoneal fluid of Balb/C mice were used as feeder cells.

The hybridoma supernatants were screened by immunoblotting on samples of the supernant of trypsin digests and the aqueous phase of Triton X-114 extracts of A6 cells using a miniblotter (Immunetics , Cambridge, MA.). Positive clones were subcloned twice by limiting dilution as described (10). Macrophages were used as feeder cells for subcloning. The hybridoma growing at the highest cloning dilution was chosen. ~ 2×10^6 cells from a hybridoma were suspended in the ice cold freezing medium (20 % FCS/20 % DMSO in RPMI medium) and frozen in styrofoam boxes at - 70 ^{o}C for one day and then transfered into liquid nitrogen.

Immunoglobulin subtyping of the mAbs was done using a kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Nitrocellulose blots of trypsin digests of A6 cells were incubated with each mAb, the mAbs were detected by incubating with rabbit anti-mouse Ig subtype, and subsequent incubation with alkaline phophatase-conjugated goat anti-rabbit IgG.

Ascites fluids were produced as described (10). Balb/C mice were primed with 2, 6, 10, 14, Tetramethyl-pentadecane (pristane; Sigma Chemicals St. Louis, MO) a week before injection of hybridoma. 1×10^7 hybridoma cells were injected into the primed mice.

9. Cell labelling and immunoprecipitation.

For overnight labelling, 90 % confluent A6 cells in 75 cm^2 flasks were incubated in 0.3 mCi ^{35}S-methionine (ICN Biomedical Inc., Irvine, CA) in 5 ml of methionine free growth medium supplemented with 5 % SCS, 10 mM Hepes, and 2 mM glutamine.

For pulse-chase experiments, A6 cells were grown to confluence in 60 mm culture dishes, and washed 3 times with 85 % PBS^+, and 2 times with methionine free and serum free growth medium. The cells were then incubated in the methionine free medium for 10 min at room temperature and then pulse labelled with 0.1 mCi ^{35}S-methionine in 1.5 ml of methionine free medium for 15 min at 28°C. To chase, cells were washed once with normal methionine containing growth medium supplemented with 5 % SCS, and incubated in 2 ml of the same medium at 28°C. Cells were extracted with ice cold immunoprecipitation buffer supplemented with protease inhibitors at the indicated times, and insoluble material was removed by centrifugation at 10,000 rpm for 30 min in a microcentrifuge.

For immunoprecipitation, mAbs 5D3, 8C2, and 19A2 were purified from the ascite fluids on a protein A-sepharose column and then coupled to CNBR activated sepharose-4B as described (36). All procedures were the same as the Con A-sepharose precipitation except that antibodies coupled to sepharose-4B were used. Immunoprecipitated polypeptides were analyzed by 8 % SDS-PAGE. For fluorography, the gel was fixed for 1 hr in 10 % acetic acid/50 % methanol, washed, and treated with 1 M sodium salicylate for 30 min

at room temperature, vacuum dryed, and exposed to X-OMAT-AR (Eastman Kodak Co., Rochester, NY) at -70°C.

10. *In vitro* maturation of oocytes and biosynthetic labelling.

The oocytes at the stage 6 were prepared as described (15). A mature female frog was anesthetized and paralyzed by incubation in ice water for 30 min and the ovary was dissected out with a pair of scissors. The sheet of ovary was cut into small pieces and most of the blood was washed away with 1 X MBSH(88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)2$, 0.41 mM $CaCl_2$, 10 mM Hepes, pH 7.4). Oocytes were dissociated from the follicle cells by the treatment with 150 mg collagenase/ovary (Sigma Chemicals, St. louis,MO). Collagenase was desalted by gel filtration and equilibrated with 1 X MBSH just before use by . Digestion of the follicle cells was done for 2-3 hr at room temperature while shaking. The completion of digestion was determined using a stereomicroscope (Wild Heerbrugg Ltd., Heerbrugg, Switzerland), by observing the disappearance of the red follicle cells attached to the oocytes. After washing off the collagenase with 1 x MBSH, large stage 6 oocytes were sorted out from small oocytes. The oocytes were allowed to settle down in the long column by gravity. When small oocytes separated from the big oocytes, they were removed through the top opening by supplying reverse water flow at the bottom of the column.

In vitro maturation of oocytes was performed as described (5). Oocytes were incubated in 5 μg progestrone (Sigma Chemicals, St. louis,MO)/ml (stock solution; 1 mg progesterone/100 % ethanol) in 1 x

MBSH for 5 hr at room temperature. The completion of maturation was confirmed by appearence of white spot on animal cap and germinal vesicle breakdown. Disappearence of the white germinal vesicle in the internal side of the animal pole was used as a criterion for the germinal vesicle breakdown. Sample oocytes were fixed in 10 % TCA for 10 min, cut by half through the animal side and the vegetal side with watch maker' forceps, and examined under the stereomicroscope. After maturation, progestrone was washed off and the oocytes were incubated in 1 x MBSH for 20 hr to determine whether maturation induced the expression of C-cadherin. As a control, oocytes were incubated in 5 µl ethanol/ml without progestrone for 5 hr.

For the biosynthetic labelling of oocytes, ~ 200 oocytes were incubated in 1 mCi of ^{35} S-methionine during the progesterone treatment. After maturation, oocytes were washed several times with 1 x MBSH, extracted with 1 % NP 40 in solution A. For immunoprecipitation with the anti-peptide polyclonal serum, the supernatant was adjusted to 2 % SDS, the proteins were heat-denatured at 96^{o}C for 5 min, and diluted to 0.2 % SDS with immunoprecipitation buffer containing no SDS. 5 µl of the polyclonal serum was added and the samples were incubated for 2 hr at 4 ^{o}C while rotating. The rabbit IgGs were precipitated by binding to protein A-sepharose. SDS-PAGE and fluorography were performed as described above.

11. Immunofluorescence staining of A6 cells and immunocytochemistry of tissues and embryos

Indirect immunofluorescence staining was performed as described (36). A6 cells grown on cover glass were fixed with methanol for 4 min and rinsed with acetone at - 20°C. 0.2 % gelatin in PBS was used as a blocking buffer. Texas red-conjugated rabbit anti-mouse IgG (Accurate Chemical & Scientific Co., Westbury, NY) was used to detect the primary mouse mAbs.

Immunocytochemistry on tissues and embryos was preformed as described (54). Cryostat sections (16 μm) were prepared from unfixed liver, lung, and embryos embedded in O.C.T compound (Miles Lab. Inc., Elkhart, IN) using an IEC microtome/cryostat (Fisher Scientific, Pittsburgh, PA), and collected on the cover glass coated with 0.2 mg/ml poly L-lysine (Sigma Chemicals, St. Louis,MO). Sections were fixed with 95 % ethanol for 15 min at 4°C. For better preservation of internal structure in later experiments, embryos were prefixed with 1 % TCA in 1 x MMR for 24 hr at 4°C as described (45). Fixed embryos were sequentially equilibrated with 5 % sucrose in PBS and 15 % Sucrose in PBS overnight at 4°C, and 7.5 % gelatin/15 % sucrose in PBS for 4 hr at 37°C. The embryos were oriented in the proper direction under the stereomicroscope and embedded in O.C.T. compound. 0.5 % BSA in PBS was used as a blocking reagent. Antibodies were applied for 1 hr and washings were done for 30 min with three changes of the blocking buffer. Samples were examined with a Zeiss epifluorescence microscope, and Tri-X film (Eastman Kodak Co., Rochester, NY) was used for photography.

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