A Cadherin-like Protein in Eggs and Cleaving Embryos of *Xenopus laevis* Is Expressed in Oocytes in Response to Progesterone

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Abstract. A new cadherin-like protein (CLP) was identified in oocytes, eggs, and cleavage stage embryos of *Xenopus laevis*. As a probe for detecting new cadherin proteins, an antiserum was raised to a 17 amino acid peptide derived from a highly conserved region in the cytoplasmic domain of all cadherins which have been sequenced to date. This antipeptide antibody recognized *Xenopus* E-cadherin and a polypeptide in *Xenopus* brain extracts similar to N-cadherin, which were independently identified by specific mAbs. In extracts of eggs and midblastula stage embryos the antipeptide antibody recognized specifically a 120-kD glycoprotein that migrated faster on SDS gels than the 140-kD E- and N-cadherin polypeptides. This 120-kD polypeptide was not recognized by the mAbs specific to E- and N-cadherin. In fact, E- and N-cadherin were not detectable in eggs or midblastula stage embryos. The possible relationship of CLP to P-cadherin, which has been identified in mouse tissues, has not yet been determined. CLP was synthesized by large, late stage oocytes. When oocytes were induced to mature in vitro with progesterone it accumulated to the same level found in normally laid eggs. It did not accumulate further to any significant extent during the early cleavage stages. CLP was detected on the surface of stage 8 blastomeres by cell surface biotinylation, but only after the tight junctions of the blastula epithelium were opened by removal of Ca^{2+}. We conclude that CLP is a maternally encoded protein that is the major, if not only, cadherin-related protein present in the earliest stages of *Xenopus* development, and we propose that it may play a role in the Ca^{2+}-dependent adhesion and junction formation between cleavage stage blastomeres.

EARLY development in amphibians begins with the formation of the blastula, a ball of cells surrounding an internal cavity that is sealed off from the outside medium by a tight surface epithelium (8). Cells of the blastula arise from a series of rapid, reductive cleavages of the egg. The development of intercellular adhesion between early blastomeres and the formation of intercellular junctions seems to occur during the extension of the cleavage furrow (5, 6, 34). In these early divisions the cleavage furrow is formed by the rapid addition of new cell surface membrane as a result of the exocytic fusion of stored intracellular vesicles (1, 34). Presumably, intercellular adhesive structures are assembled from components stored in the egg as cleavage proceeds. Therefore, cleaving amphibian embryos provide a means to investigate the biogenesis and regulation of intercellular junctions occurring during the normal development of a simple epithelial tissue.

All of the cells of the *Xenopus* blastula are linked together by a Ca^{2+}-dependent adhesion mechanism (24). In the systems studied so far, the proteins underlying Ca^{2+}-dependent intercellular adhesion have been found to belong to the cadherin family of adhesion molecules, including E-cadherin in epithelial tissues, N-cadherin in neural tissues, and P-cadherin in placenta (9, 35). In early mammalian embryos the first cadherin to be expressed is the epithelial cadherin, E-cadherin (also known as uvomorulin) (32, 39), and in the chick it is L-CAM (36), which is generally believed to be the same as E-cadherin. Since the *Xenopus laevis* blastula forms a tight epithelium, it was expected that E-cadherin would be the first cadherin expressed, and that it would be stored in the egg ready for recruitment into the adhesive surfaces of the cleaving blastomeres. Surprisingly, we found that *Xenopus* E-cadherin was not detectable in eggs or early cleaving embryos (2). 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 therefore asked whether another member of the cadherin family of adhesion molecules, perhaps one that has not yet been identified, is expressed in the egg, the early cleavage stages, and the blastula of *Xenopus laevis*. The amino acid sequences of several different cadherin types in different species have been determined from cDNA clones (7, 14, 28). The cytoplasmic tail domain has regions of very high homol-

ogy between the different cadherin types and between the same cadherin in different species. We therefore used antibodies raised to one of these conserved regions as a means to detect and identify cadherins in *Xenopus* eggs and early embryos.

Materials and Methods

Preparation of Antigen and Generation of Antipeptide Antibody

A peptide corresponding to amino acid residues 665–681 derived from the chicken L-CAM cDNA sequence (7) with the addition of an NH_2-terminal cysteine residue was designed by Gene Napolitano and Louis Reichardt at University of California, San Francisco and Sarah Crittenden at the University of Wisconsin (Madison, WI) and generously provided to us to make the antipeptide antibody. The peptide was conjugated to the carrier protein, keyhole limpet hemocyanin, as described (18) (5 mg peptide/4.5 mg carrier protein) except that Sulfo-MBS (*m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester) (Pierce Chemical Co., Rockford, IL) was used instead of MBS. With this reagent the conjugation could be carried out entirely in aqueous solution.

A rabbit was immunized intradermally with the conjugated peptide in complete Freund's adjuvant in an amount equivalent to 0.5 mg of the free peptide. It was boosted 3 wk later with 0.5 mg peptide equivalents in incomplete Freund's adjuvant injected subscapularly, another 3 wk later intramuscularly with 0.5 mg peptide equivalents in PBS, 10 d later intramuscularly with 125 μg peptide equivalents in PBS, and on days 1 and 2 thereafter with 125 and 250 μg peptide equivalents intravenously in PBS.

Metabolic Cell Labeling, Tissue Sample Preparation, and Immunoprecipitation

A6 cells were cultured and labeled with [^{35}S]methionine as described (2). The cells were extracted with immunoprecipitation buffer (1% Triton X-100, 0.5% Na deoxycholate, 0.2% SDS, 0.15 M NaCl, 20 mM Hepes pH 7.4) and supernatants were saved after centrifugation in a microcentrifuge at 10,000 rpm for 30 min. The supernatants were adjusted to 2% SDS, incubated for 30 min at room temperature, diluted to 0.2% SDS with immunoprecipitation buffer containing no SDS, and immunoprecipitated with 3 μl of the antipeptide or the preimmune serum. Antibody–antigen complexes were precipitated with protein A–Sepharose as described (2). SDS-PAGE and fluorography were performed as described (2, 10).

Extracts of *Xenopus* tissues, including skin, brain, pericardium, and peritoneum were extracted as described (2).

Handling of Xenopus Eggs and Embryos and Preparation of Samples

Production of eggs, fertilization and removal of the jelly coat were done as described (22) with minor modification. To obtain large numbers of embryos, frogs were allowed to lay eggs directly into MMR[1] (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) before fertilization. Fertilization efficiency under these conditions was ~90%. Embryos were staged according to the normal table of *Xenopus laevis* development (23).

Dejellied eggs or embryos were extracted with a 1:1 vol of 1% NP-40 in solution A (10 mM Hepes pH 7.4, 150 mM NaCl, 1.5 mM EDTA) supplemented with protease inhibitors (1 mM PMSF, 0.5 mM iodoacetamide, 1 μg/ml pepstatin A, 2 μg/ml leupeptin, 4 μg/ml aprotinin, 10 μg/ml antipain, 50 μg/ml benzamidine, 10 μg/ml soybean trypsin inhibitor) as described (2). The glycoproteins in the supernatant were enriched by binding to Con A–Sepharose-4B before immunoblotting assay.

Cell Surface Labeling

Cell surface labeling of embryos with biotin was carried out by a modification of a published procedure (20, 31). NHS-LC-Biotin (sulfosuccinimidyl 6-(biotinamido) hexanoate; Pierce Chemical Co.) was used instead of Sulfo-NHS-biotin (sulfosuccinimidobiotin) because it dissolved readily in aqueous solution without prior preparation as a stock solution in dimethyl sulfoxide. Labeling was done on ice for 30 min in 1 mg/ml NHS-LC-Biotin in the appropriate buffer (below). Labeling was quenched by rinsing embryos or blastomeres three times in buffer containing 10 mM glycine.

To label embryos, the jelly coat was removed after fertilization and embryos were transferred into a 1:10 concentration of MMR to allow development to proceed. A little over 200 embryos were labeled in each condition. Fertilized eggs that had raised fertilization envelopes but had not yet begun to cleave were labeled in MMR. Intact blastulae at stage 8 were transferred to MMR at room temperature for 20 min and then labeled in MMR. Another set of blastulae were rinsed three times and incubated for 20 min at room temperature in a Ca^{2+}-free buffer (0.1 M Na isethionate, 20 mM Na pyrophosphate, 20 mM glucose, pH 8.5) to open the tight junctions and allow access of labeling reagent to the internal blastomere cell surfaces. Because blastomeres were constrained by the fertilization envelope, they did not dissociate completely as previously reported (22), but were observed under the stereomicroscope to become somewhat rounded. Labeling was performed in the Ca^{2+}-free buffer adjusted to pH 7.8. As a control another set of embryos was lysed directly into Ca^{2+}-free buffer containing 1 mg/ml NHS-LC-biotin by repeated pipeting through a 1-ml pipet tip. In this case it was not possible to quench the reaction by rinsing in 10 mM glycine.

After labeling and quenching was complete, embryos or blastomeres were extracted with a 2:1 volume of 1% NP-40 in solution A with the protease inhibitors and cadherin-like protein (CLP) was immunoprecipitated with the antipeptide antibody (overnight incubation) as described above. Biotinylated proteins were detected after their separation by SDS-PAGE and transfer to nitrocellulose by blotting with alkaline phosphatase-conjugated avidin using a Vectastain kit from Vector Laboratories (Burlingame, CA).

In Vitro Maturation and Biosynthetic Labeling of Oocytes

A mature female frog was anesthetized with 0.3% ethylaminobenzoate in the bath water and its ovary was dissected out. Oocytes were prepared as described (4). The sheet of ovary was cut into small pieces and most of the blood was washed away with modified Barth's saline (11) containing 10 mM Hepes instead of Tris as the buffer (MBSH). Oocytes were dissociated from follicle cells by treatment with 150 mg type IA collagenase/ovary (Sigma Chemical Co., St. Louis, MO). The collagenase was first desalted and equilibrated with MBSH before use. Digestion of follicle cells occurred for 2–3 h at room temperature with gentle shaking. The completion of digestion was determined by looking in a stereomicroscope for the disappearance of the red-colored follicle cells from the surface of the oocytes. After washing off collagenase with MBSH, large oocytes were separated from small oocytes by allowing them to settle against an upward flow of medium.

In vitro maturation of oocytes was performed as described (4). Oocytes were incubated in 5 μg progesterone (Sigma Chemical Co.) per milliliter in MBSH (added from a stock solution of 1 mg progesterone/ml ethanol) for 5 h at room temperature. The completion of maturation was determined by the appearance of a white spot on the animal cap and by germinal vesicle breakdown. Disappearance of the germinal vesicle within the internal side of the animal pole was determined using a stereomicroscope after fixing a sample of oocytes in 10% TCA for 5 min and cutting them in half horizontally with a watchmaker's forceps. After maturation, oocytes were incubated in progesterone-free MBSH for different periods of time for up to 20 h. Control oocytes were incubated in 5 μl ethanol/ml MBSH for 5 h.

For the biosynthetic labeling of oocytes, ~100 oocytes were incubated in 1 mCi of [^{35}S]methionine during the progesterone treatment. After maturation, oocytes were washed several times with MBSH, extracted with 1% NP-40 in solution A containing the protease inhibitors and immunoprecipitated with the antipeptide antibody as described above.

Anticadherin Antibodies and Immunoblotting

A mixture of four independent mAbs against *Xenopus* E-cadherin, described previously (2), were used in all immunoblotting experiments. Anti-*Xenopus* E-cadherin mAbs 8C2 and 5D3 were subclass IgG1, while mAbs 19A2 and 31D2 were subclass IgG2b. mAbs 5D3 and 19A2 recognize both the $+Ca^{2+}$ and the $-Ca^{2+}$ conformations of E-cadherin, while mAbs 8C2 and 31D2 recognize only the $-Ca^{2+}$ conformation (2). The mAb NCD-2 to chicken N-cadherin (12) was a gift of M. Takeichi (Kyoto University, Kyoto, Japan). Alkaline phosphatase–conjugated goat anti-rabbit IgG and anti-mouse IgG (Bio-Rad Laboratories, Richmond, CA), and alkaline

1. *Abbreviations used in this paper*: CLP, cadherin-derived protein; MBSH, modified Barth's saline containing 10 mM Hepes instead of Tris as the buffer; 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.

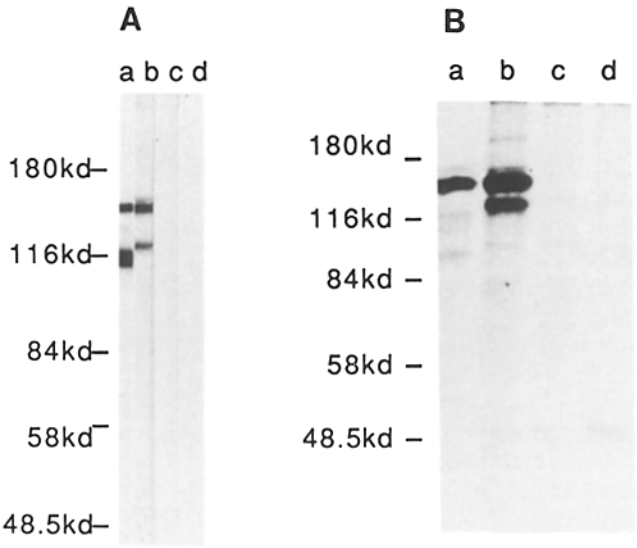

Figure 1. Recognition of polypeptides in A6 cells by the anti-cadherin peptide antibody. (*A*) Immunoblot analysis of SDS extracts of A6 cell. Anti-E-cadherin mAb (lane *a*); antipeptide antibody (lane *b*); antipeptide antibody preincubated with 30 μg cadherin-derived peptide (lane *c*); preimmune serum (lane *d*). (*B*) Immunoprecipitation of extracts of [^{35}S]methionine-labeled cells. Anti-E-cadherin mAb (lane *a*); antipeptide antibody (lane *b*); antipeptide antibody preincubated with 30 μg cadherin-derived peptide (lane *c*); preimmune serum (lane *d*).

phosphatase-conjugated rabbit anti-rat IgG (Miles Laboratories Inc., Elkhart, IN) were used as second antibodies to detect rabbit, mouse, and rat antibodies, respectively. SDS-PAGE and immunoblotting were performed as described (2, 10). Proteins were separated on 8% polyacrylamide/0.21% bis-acrylamide gels under denaturing conditions. Prestained molecular mass markers (Sigma Chemical Co.) were used for the molecular mass estimation of proteins. Proteins were electrophoretically transferred to nitrocellulose and immunoblotted.

Results

Recognition of Xenopus Cadherins by the Antipeptide Antibody

An antiserum raised in rabbits against a peptide corresponding to amino acid residues 665–681 derived from the chicken LCAM cDNA sequence (7) was found to recognize cadherin polypeptides in cells and tissues from *Xenopus laevis*. In immunoblot analysis of extracts of the A6 *Xenopus* epithelial cell line (Fig. 1 *A*) this antiserum bound with high titer to a 140-kD polypeptide (lane *b*) as do mAbs to E-cadherin (lane *a*). A major 116-kD degradation product of E-cadherin was recognized by the mAbs to E-cadherin, which bind to the ectoplasmic domain of the protein (Fig. 1 *A*, lane *a*), but was not recognized by the antipeptide antibody (Fig. 1 *A*, lane *b*). Presumably this fragment had been cleaved away from the cytoplasmic tail, which harbors the epitopes recognized by the antipeptide antibody.

The specificity of the binding of the antipeptide antibody to the 140-kD polypeptide was demonstrated by the absence of binding of the preimmune serum (Fig. 1 *A*, lane *d*) and by the inhibition of the binding of the immune serum after it was incubated with an excess of the cadherin-derived peptide against which it was made (lane *c*). The antibody also immunoprecipitated a biosynthetically labeled 140-kD polypeptide from detergent extracts of A6 cells (Fig. 1 *B*, lane *b*) just like the mAbs to E-cadherin (lane *a*). Again, the E-cadherin-sized polypeptide was not recognized by preimmune serum or by the antipeptide antibody treated with excess peptide (Fig. 1 *B*, lanes *c* and *d*, respectively).

A polypeptide of ~120 kD not recognized by the mAbs to E-cadherin was recognized by the antipeptide antibody, both by immunoblotting and by immunoprecipitation (Fig. 1 *A*, lane *b*, and *B*, lane *b*). The reactivity was specifically inhibited by excess cadherin-derived peptide, and therefore is likely to contain portions of the cadherin cytoplasmic tail sequence. It is possible that this polypeptide is a cadherin gene product distinct from E-cadherin which is coexpressed in the A6 cell line (see Discussion).

Fig. 2 demonstrates that the antipeptide antibody recognizes the very same 140-kD polypeptide as mAbs to E-cadherin. When E-cadherin was immunoprecipitated with the anti-E-cadherin mAbs it was recognized with the antipeptide antibody by immunoblotting (Fig. 2, lane *a*). Conversely, when immunoprecipitation was first carried out with the antipeptide antibody, E-cadherin was detected by immunoblotting with the mAbs (Fig. 2, lane *c*). E-cadherin was not detected by immunoblotting with either antibody when preimmune serum was used in the immunoprecipitation step (Fig. 2, lanes *b* and *d*). Therefore, the antipeptide antibody specifically reacted with E-cadherin of *Xenopus laevis*. Re-

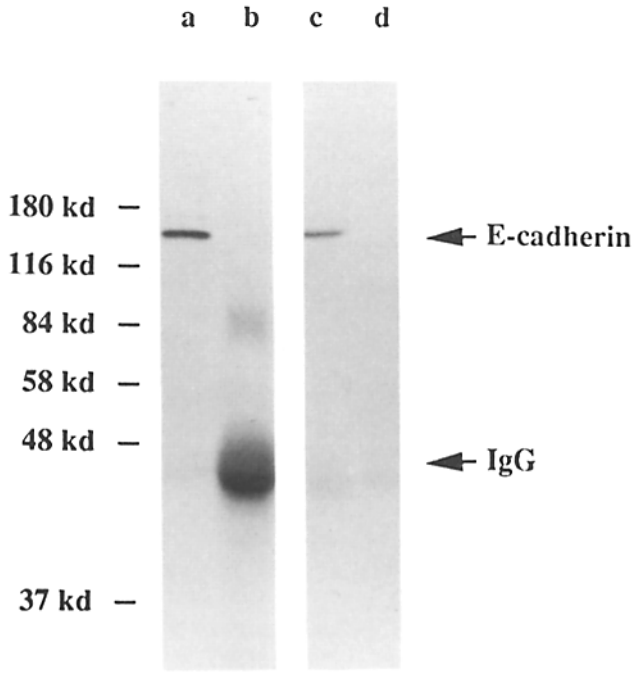

Figure 2. Antipeptide antibody and mAbs to *Xenopus* E-cadherin recognize the same polypeptide in A6 cells. Extracts of unlabeled A6 cells were immunoprecipitated with either a mAb to *Xenopus* E-cadherin (lane *a*), the antipeptide antibody (lane *c*), or with preimmune serum (lanes *b* and *d*). After separation on an SDS gel the immunoprecipitates were analyzed by immunoblotting either with the antipeptide antibody (lanes *a* and *b*) or with the mAb to E-cadherin (lanes *c* and *d*).

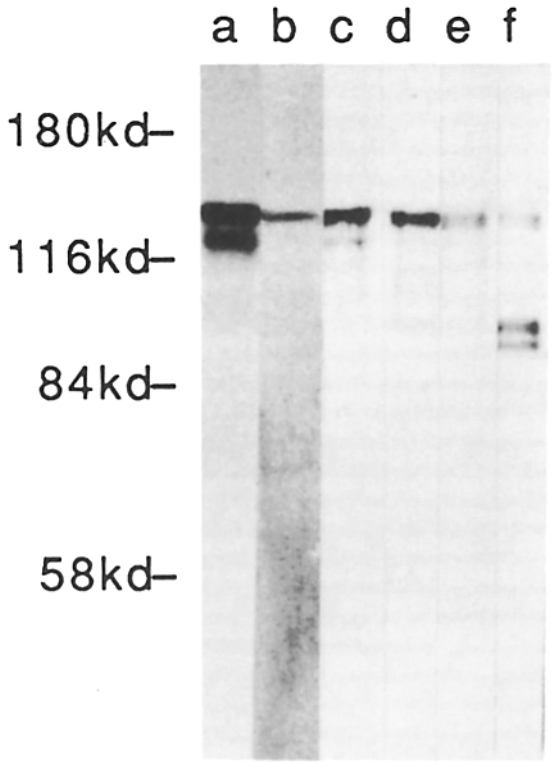

Figure 3. Antipeptide antibody recognizes cadherins in other tissues. Extracts of brain (lanes *a* and *b*), peritoneum (lanes *c* and *d*), and skin (lanes *e* and *f*) were immunoblotted with 1:500 dilution of the polyclonal serum against the peptide (lanes *a*, *c*, and *e*), with the mAb NCD-2 against chicken N-cadherin (lane *b*), and with the hybridoma supernatants against *Xenopus* E-cadherin (lanes *d* and *f*). Numbers at left are molecular mass markers in kilodaltons.

activity with canine E-cadherin in MDCK cells was also observed (not shown).

The epitope formed by the conserved cytoplasmic domain represented by the peptide seems to be masked in the "native" state and requires denaturation to be recognized by the antipeptide antibody. Despite the high level of reactivity of the antipeptide antibody against denatured E-cadherin, the antibody staining of E-cadherin could not be detected in A6 cells by indirect immunofluorescence (not shown). This contrasts the intense staining of E-cadherin observed in A6 cells using the mAbs directed against the ectoplasmic domain of the protein (2). Similarly, the antipeptide antibody could not immunoprecipitate E-cadherin from nonionic detergent extracts of A6 cells (not shown). For immunoprecipitation (Fig. 1 *B*) it was necessary to first denature the cell extract in SDS. It is possible that this conserved amino acid sequence is masked by the binding of the "catenins," three cytoplasmic polypeptides that form a protein complex with E-cadherin by binding to its cytoplasmic tail (26).

The antipeptide antibody probably also recognizes other cadherin polypeptides in tissues of *Xenopus laevis* (Fig. 3, lanes *a*, *c*, and *e*). N-cadherin was identified as a 140-kD polypeptide in extracts of *Xenopus* brain with mAb NCD-2 to chicken N-cadherin (Fig. 3, lane *b*). The same-sized polypeptide in *Xenopus* brain was detected by the antipeptide antibody (Fig. 3, lane *a*). Since significant amounts of E-cadherin were not found previously in *Xenopus* brain (2), it is probable that the 140-kD polypeptide recognized by the antipeptide antibody is N-cadherin. In addition to the 140-kD polypeptide, the antipeptide antibody also bound a 120-kD polypeptide in brain that was not detected by mAb NCD-2.

An attempt was made to determine whether the antipeptide antibody recognized P-cadherin in *Xenopus*. Unfortunately, it is not yet possible to unambiguously detect P-cadherin in *Xenopus* tissues, because cross-reacting antibodies are not available and because *Xenopus* does not have a placenta, the major source of P-cadherin in mouse (25). In the mouse, P-cadherin is also expressed in the mesothelium as well as in the epidermis (15, 17), and therefore these tissues were examined as a potential source of P-cadherin in *Xenopus* (Fig. 3, lanes *c–f*). In a combined extract of the peritoneal membrane and the pericardium as a source of mesothelium, the antipeptide antibody (Fig. 3, lane *c*) and the anti–E-cadherin mAb (lane *d*) both immunoblotted a major polypeptide of 140 kD. This reactivity probably represents the presence of E-cadherin in these tissues. Like A6 cells and brain, a minor 120-kD polypeptide was detected by the antipeptide antibody but not by the mAbs to E-cadherin. In an extract of *Xenopus* skin both antibodies recognize a 140-kD polypeptide (Fig. 3, lanes *e* and *f*), which again is probably due to the presence of E-cadherin. The lower molecular mass bands in Fig. 3, lane *f* are probably degradation products of E-cadherin in skin. Because these tissues express high levels of E-cadherin it is questionable to use them as

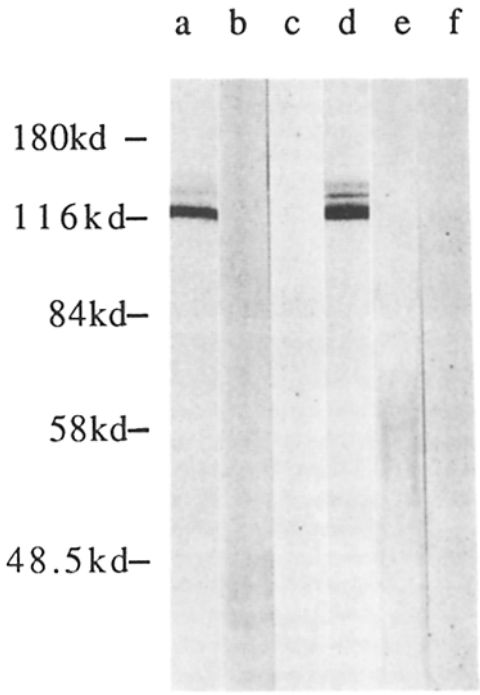

Figure 4. Identification of a novel CLP in *Xenopus* eggs and embryos. Extracts of ~200 *Xenopus* eggs (lanes *a–c*) and embryos at midblastula stage (lanes *d–f*) were enriched for glycoproteins by binding to Con A–Sepharose and then immunoblotted with the antipeptide antibody against the cadherin-derived peptide at a dilution of 1:500 (lanes *a* and *d*); a monoclonal antibody against chicken N-cadherin (NCD-2) (lanes *b* and *e*); and a mixture of monoclonal antibodies (5D3, 8C2, and 19A2) against *Xenopus* E-cadherin (lanes *c* and *f*). Numbers at left are molecular mass markers in kilodaltons.

a specifically enriched source of P-cadherin. The minor 120-kD band in peritoneum recognized by the antipeptide antibody is a possible candidate, but amino acid sequence data or a specific antibody will be necessary to definitively identify *Xenopus* P-cadherin in any tissue.

Identification of a CLP in Oocytes, Eggs, and Cleaving Embryos

To determine whether a cadherin protein exists that could account for the Ca^{2+}-dependent adhesion between early *Xenopus* blastomeres, glycoprotein enriched fractions of extracts of eggs and cleavage staged embryos were analyzed by immunoblotting with the various anticadherin antibodies. The antipeptide antibody effectively detected a major polypeptide of 120-kD in both eggs and midblastula stage embryos (Fig. 4). It also recognized variably minor polypeptides of slightly higher molecular mass. None of these polypeptides were detected with the preimmune serum (not shown) or when the antipeptide antibody was incubated with an excess of the cadherin-derived peptide (Fig. 6). Therefore, the major 120-kD polypeptide and minor higher molecular mass bands contain an epitope, and presumably an amino acid sequence, in common with all the known cadherins.

The 120-kD CLP and higher molecular mass polypeptides in eggs and midblastula embryos do not seem to be either E- or N-cadherin. As observed previously (2), neither eggs nor midblastula stage embryos contained detectable amounts of E-cadherin (Fig. 4, lanes *c* and *f*). The absence of detectable E-cadherin epitopes could not be attributable to proteolytic degradation, because exogenous E-cadherin from A6 cells added to egg homogenates could be completely recovered in immunoblots (data not shown). Also, mAb NCD-2, which recognizes N-cadherin (Fig. 3), failed to recognize the 120-kD cadherin or the minor higher molecular mass polypeptides (Fig. 4, lanes *b* and *e*). Whether the variably observed higher molecular mass polypeptides are precursors or modifications of CLP, or represent additional minor cadherin gene products remains to be determined.

We wished to know whether the time of expression of CLP is consistent with it having a function in the egg and/or cleaving embryo, rather than, for example, being a remnant left over from the interaction between the oocyte and the follicle cells. Oocytes were prepared from follicular tissue by collagenase treatment to insure that the follicle cells had been removed. In immunoblots of extracts of large, late stage oocytes that had been incubated overnight in vitro in saline, CLP was present at low but detectable levels (Fig. 5 *A*, lane *a*). When the same batch of oocytes was induced to mature with 5-h progesterone treatment and then incubated overnight in vitro, the CLP polypeptide accumulated to the same levels (Fig. 5 *A*, lane *b*) as it is found in naturally laid eggs (lane *c*). The biosynthesis of the CLP polypeptide by oocytes was confirmed by immunoprecipitating a denatured extract of oocytes that had been labeled with [^{35}S]methionine for 5 h (Fig. 5 *B*). A biosynthetically labeled 120-kD polypeptide was immunoprecipitated by the antipeptide antibody (Fig. 5 *B*, lanes *a* and *c*), but not in the presence of excess cold cadherin-derived peptide (lane *b*). These results show that CLP is synthesized by late stage oocytes, and that its accumulation to the levels found in the egg is dependent on maturation induced by progesterone.

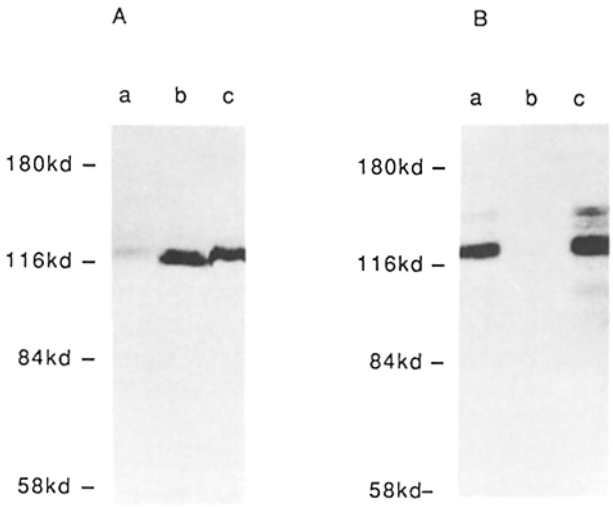

Figure 5. CLP expression in oocytes and dependence on progesterone-induced maturation. (*A*) Levels of CLP expression in progesterone-treated and untreated oocytes at 20 h maturation in vitro. Oocytes were incubated for 5 h with (lane *b*) or without (lane *a*) 5 μg/ml progesterone and subsequently incubated in MBSH free of progesterone for 20 h. About 200 oocytes from each were extracted and immunoblotted with a 1:500 dilution of the antipeptide antibody. For comparison an extract of 200 eggs was also immunoblotted with the same serum (lane *c*). (*B*) Biosynthetic labeling of CLP polypeptides. Oocytes were labeled with 1 mCi of [^{35}S]methionine for 5 h with (lane *c*) or without (lane *a*) progesterone. Extracts were SDS- and heat-denatured, and immunoprecipitated with the antipeptide antibody (lanes *a* and *c*) or with the antipeptide antibody after preincubation with 30 μg cadherin-derived peptide (lane *b*).

Progesterone did not greatly increase the extent of biosynthetic labeling of CLP during the first 5 h of treatment (Fig. 5 *B*, lane *c* vs. lane *a*). By immunoblot analysis CLP was observed to accumulate gradually overnight after progesterone induction (data not shown). The accumulation of CLP during overnight incubation could result either from a gradual increase in its biosynthesis after completion of the 5-h maturation process or from a decrease in its rate of turnover.

Intercellular adhesion first becomes required in *Xenopus* embryogenesis during the cleavage stages (24). Therefore, it was of interest to determine whether the levels of CLP changed during the cleavage stages of development. By the criterion of semiquantitative immunoblotting, the levels of CLP increased only slightly during the cleavage stages (Fig. 6, lanes *a–e*). The higher molecular mass minor polypeptides became more prominent during cleavage. Their significance is not clear because their relationship to CLP is unknown. These findings along with the oocyte biosynthesis data demonstrate that the time of greatest CLP accumulation is during the maturation of the oocyte to the egg, with little change in levels occurring during the early cleavage stages.

Localization of CLP in Eggs and Blastulae

If CLP is involved in adhesion between early blastomeres, it should be expressed at the blastomere surface, but not present at the outer cortical membrane surface of the egg. Cell surface localization was analyzed by labeling with the membrane impermeable protein biotinylating reagent, NHS-

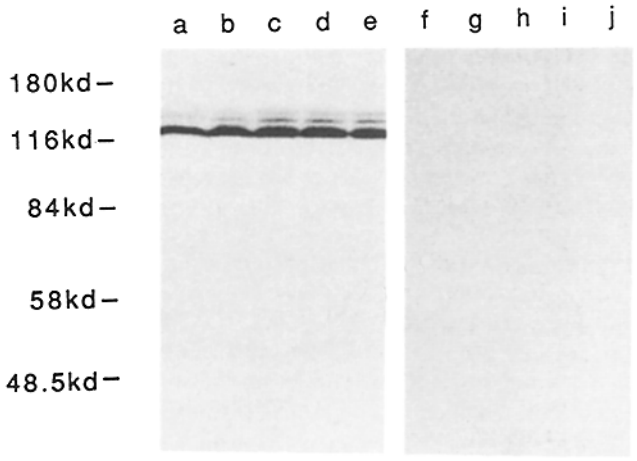

Figure 6. CLP expression during early cleavage stage development of *Xenopus laevis*. Extracts of ~200 eggs (lanes *a* and *f*) or embryos at 2 (lanes *b* and *g*), 4 (lanes *c* and *h*), 6 (lanes *d* and *i*), and 7 h (lanes *e* and *j*) after fertilization were immunoblotted with 1:500 dilution of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

LC-biotin (20, 31). Embryos were labeled with biotin through their fertilization envelopes (Fig. 7). Either fertilized eggs (Fig. 7, lanes *a* and *a'*), intact stage 8 blastulae (lanes *b* and *b'*), or stage 8 blastulae with opened intercellular junctions (lanes *c* and *c'*) were labeled. Detergent extracts of embryos were immunoprecipitated with the antipeptide antibody (Fig. 7, lanes *a*, *b*, *c*, and *d*) or preimmune serum (lanes *a'*, *b'*, *c'*, and *d'*), and biotinylated proteins were detected by blotting with avidin. Significant labeling of a 120-kD polypeptide occurred in blastulae that had been incubated in Ca^{2+}-free medium to open the tight junctions of the surface epithelium (Fig. 7, lane *c*). The absence of this polypeptide in the preimmune control (Fig. 7, lane *c'*) indicates that it represents labeling of CLP.

Greatly reduced labeling of CLP was observed in blastulae with intact tight junctions (Fig. 7, lane *b*). No labeling of CLP was detected when fertilized eggs, before first cleavage, were biotinylated (Fig. 7, lane *a*). The reduction in CLP labeling in these samples was not due to poor biotinylation, because the total Con-A-binding glycoproteins of the three samples were biotinylated to similar extents (data not shown). As an additional control, embryos were intentionally lysed in the labeling medium by vigorous pipetting (Fig. 7, lanes *d* and *d'*). Little, if any, CLP was labeled in lysed embryos. These results together demonstrate that CLP was labeled in Ca^{2+}-free treated blastulae due to its expression at the blastomere surface, and not due to artifactual labeling after cell lysis or from permeation of the reagent through the plasma membrane. Moreover, these results suggest that CLP in the egg is present in an intracellular pool, and when it becomes expressed at the blastomere plasma membrane, it is localized to the internal cell surfaces that face the blastocoel cavity.

Discussion

The antipeptide serum used in this study seems to be a good probe for detecting a wide variety of cadherins in different species. Although raised to a peptide sequence derived from the known chicken and mouse cadherins, it specifically recognized cadherins in tissues of *Xenopus laevis*. The antiserum bound to E-cadherin in extracts of cultured A6 cells, and of *Xenopus* skin and peritoneum; it also bound to a polypeptide in *Xenopus* brain extracts that had the same molecular size as N-cadherin. The specificity of the antiserum for the cadherin sequence was readily demonstrated by the inhibition of its binding to E-cadherin with an excess of the peptide against which it was raised. These findings suggest that the antipeptide antibody is capable of recognizing members of the cadherin family of proteins in tissues of *Xenopus laevis*. Similar conclusions have been reached from observations by L. Reichardt, J. Lilien, and their co-workers examining independently derived antisera to the same peptide (Reichardt, L., personal communication). Therefore, this antibody might be expected to be a suitable reagent for the identification of new members of the cadherin family of proteins in *Xenopus*.

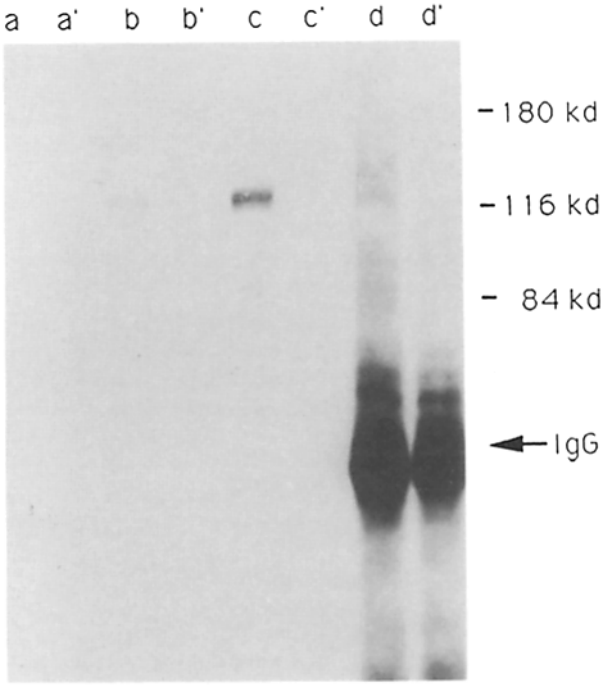

Figure 7. Cell surface biotinylation of CLP in *Xenopus* embryos. Lanes *a* and *a'*, embryos were biotinylated 1 h after fertilization, after the raising of the fertilization envelope but before first cleavage; lanes *b* and *b'*, stage 8 embryos were biotinylated in Ca^{2+}-containing medium to keep tight junctions intact; lanes *c* and *c'*, stage 8 embryos were incubated in Ca^{2+}-free buffer at room temperature before biotinylation in order to open the tight junctions; lanes *d* and *d'*, stage 8 embryos were lysed in Ca^{2+}-free buffer containing the biotinylation reagent. The IgG contains so much biotin because the unquenched NHS-LC-biotin remained during the immunoprecipitation and because it is the major protein recovered in the immunoprecipitate. For each sample, ~200 μl of labeled embryos were extracted with detergent-containing buffer, of which half was immunoprecipitated with the antipeptide antibody (*a*, *b*, *c*, and *d*) and half with the preimmune serum (*a'*, *b'*, *c'*, and *d'*). Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose, and biotin-containing proteins were detected with alkaline phosphatase–conjugated avidin.

We have used this antibody to detect a CLP of unknown identity in oocytes, eggs, and cleavage stage embryos. CLP seems to be the major, if not only, protein expressed in cleavage stage embryos that harbors the highly conserved cadherin sequence recognized by this antibody. Because neither E-cadherin nor N-cadherin are expressed at these stages, CLP is a very good candidate to be the cadherin that mediates Ca^{2+}-dependent adhesion in the blastula. Unfortunately, it has not yet been possible to use another established criterion for identifying cadherins, the characteristic protection of the ectoplasmic domain from trypsin degradation by the presence of calcium ions (10, 16, 33, 38). In the absence of Ca^{2+} the ectoplasmic domain is extensively degraded, but in the presence of Ca^{2+} most of it is cleaved from the cell surface in a soluble form that is resistant to further proteolysis. It has not been possible to use the antipeptide antibody, which recognizes the cytoplasmic tail domain, to distinguish between degradation or release of the ectoplasmic domain. However, because CLP was detected with an antibody raised against a specific cadherin consensus sequence that recognizes known cadherins in *Xenopus* tissues, we believe that it is likely to be a maternally encoded member of the cadherin family of proteins. Eventually the amino acid sequence of CLP determined from its cDNA clone will reveal its overall relatedness to the other cadherins.

The presence of CLP in eggs and cleaving embryos could account, at least in part, for the Ca^{2+}-dependent adhesion system that has been shown to be present in early *Xenopus* blastomeres (24). However, we do not yet have experimental evidence that this protein functions in adhesion between early blastomeres. The usual adhesion assays have not yet been performed because the antipeptide antibody recognizes the cytoplasmic tail of CLP only under denaturing conditions. However, several properties of CLP are consistent with it being an adhesion molecule. It is a glycoprotein, since it binds to the lectin Con-A, and it is expressed at the cell surface, since it can be labeled by a membrane impermeable reagent. The inaccessibility of CLP to surface labeling in intact blastulae indicate that it is expressed on the internally disposed surface of the blastomeres, which would be expected to participate in cell-to-cell contacts. To determine whether CLP participates in Ca^{2+}-dependent adhesion events, additional antibodies raised to the extracellular portion of the protein will again be required.

The increase in CLP expression during meiotic maturation is most likely an indirect posttranscriptional 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) (21, 29). The increased expression of oocyte proteins during maturation results from translational regulation of stored maternal mRNAs by a mechanism involving mRNA recruitment onto polysomes (27). The early induction of a small number of proteins, including the c-*mos* protooncogene, is required for meiotic maturation and GVBD (30). Quantitatively, however, the greatest hormone-induced increase in protein synthesis occurs after GVBD (21, 27). These "late" proteins, including for example the histones, are believed to be destined to function in later stages of development. CLP 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-h period of progesterone treatment. Although we have not ruled out regulation of CLP 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. Therefore, it is likely that CLP plays a role in the egg and/or early stages of development before the onset of zygotic gene transcription.

It is possible that CLP is a new member of the cadherin family distinct from all other previously identified cadherins. In the present study we have demonstrated that CLP is distinct from E-cadherin and N-cadherin by its molecular size and by its lack of reactivity with mAbs to E- and N-cadherin. Moreover, it is not a degradation product of E-cadherin, the first cadherin species to be expressed in other organisms (2). It is possible, however, that CLP is the *Xenopus* equivalent of P-cadherin, since it has not yet been possible to identify P-cadherin in *Xenopus*. It is also conceivable that CLP corresponds to a protein in *Xenopus laevis* that is recognized by antibodies to chicken L-CAM. In contrast to mAbs to *Xenopus* E-cadherin (2), antibodies to chicken L-CAM detected a polypeptide of ~120 kD in *Xenopus* skin and a very weak immunohistological reactivity in blastula stage embryos (19). If CLP is distinct from the other known cadherin proteins, it could either be the product of a distinct cadherin gene or arise from posttranscriptional processing of a known cadherin gene. To determine the definitive relationships between CLP and other known cadherins the generation of additional antibodies specific to *Xenopus* CLP and/or the cloning of several of the *Xenopus* cadherin cDNAs will be required.

CLP may be present along with other cadherins in tissues other than the egg or the blastula. In the A6 kidney cell line, the brain, and perhaps mesothelial tissues, the anticytoplasmic tail peptide antibody specifically recognized polypeptides of ~120 kD that were not recognized by mAbs to E-cadherin or N-cadherin. The similarity of their molecular sizes on SDS gels to CLP raises the possibility that they are all the same protein. Such a conclusion is premature, however, because the antipeptide antibody has a broad specificity for most, if not all, cadherins. Coexpression of two different cadherins in one cell type would not be surprising, since the expression of multiple cadherins has been observed, especially in developing tissues (13, 15, 17, 35).

In some experiments minor bands of a slightly lesser mobility on SDS gels were resolved from the major 120 kD CLP polypeptide. These polypeptides might represent biosynthetic precursors or posttranslational modifications of CLP. Larger biosynthetic precursors having an NH_2-terminal propeptide have been observed for all the known cadherins (2, 3, 14), and at least E-cadherin and L-CAM are known to be phosphorylated on serine residues (3, 37, 40). Again, the broad specificity of the antipeptide antibody makes it difficult to know the relationship between all of these polypeptides. It is clear, however, that these bands are not recognized by mAbs to E- and N-cadherin. Specific probes for CLP and pulse–chase metabolic labeling experiments will enable us to examine whether they are metabolically related to CLP, or whether they represent yet additional cadherin species in *Xenopus*.

We propose that CLP 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 CLP is synthesized by the oocyte during its maturation to a fertilizable egg and is stored in the egg for use during early development. Preliminary experiments indicate that CLP is present in the egg in intracellular vesicles (unpublished observations). Recruitment of CLP-containing vesicles to the internal blastomere surfaces could occur by their fusion with the advancing cleavage furrow. Fusion of stored vesicles with the advancing cleavage furrow has been observed by electron microscopy (1, 34). Further experiments on the structure and function of CLP and the mechanism of its recruitment into the blastomere cell surface may yield insights into the biogenesis and regulation of adhesive junctions during embryonic development.

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