Identification of two structural types of calcium-dependent adhesion molecules in the chicken embryo

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ABSTRACT By using an immunological and peptide mapping approach two calcium-dependent cell–cell adhesion molecules (calCAMs) in the embryonic chicken are compared. A third closely related molecule is identified and compared to the two calCAMs. One of the calCAMs appears to be identical to the previously identified adhesion molecule N-cadherin, originally identified in chicken retina and localized to neural tissues. The second is the same as L-CAM, originally identified in chicken liver but localized to a variety of epithelial tissues. The third, also found in liver, is similar to L-CAM but is much closer in structure to N-cadherin. It is, however, immunologically distinct from N-cadherin. We therefore refer to this newly identified molecule as CRM-L, for cadherin-related molecule in liver. CRM-L, N-cadherin, and L-CAM are all cell-surface proteins with a similar stability to tryptic digestion in the presence of calcium. CRM-L has the same molecular mass and isoelectric point as N-cadherin but is distinct from L-CAM in these properties. Two-dimensional peptide maps of complete tryptic digests reveal that CRM-L shares 69% of its peptides with N-cadherin and 20% with L-CAM. On the basis of these data, we suggest that there are at least two distinguishable types of calCAMs in the chicken embryo: one represented by the closely related molecules N-cadherin and CRM-L, and another represented by L-CAM.

Both calcium-independent cell-adhesion molecules (CAMs), such as neural CAM (N-CAM) (1) and calcium-dependent CAMs (cal-CAMs) are thought to play important roles in morphogenesis. Expression of the calCAMs has been shown to be regulated both temporally and spatially throughout the development of an organism by immunohistochemical studies (2–7). In addition, perturbation studies with antibodies to these molecules implicate them in defined morphogenetic events such as blastocyst compaction (8–11), neurite outgrowth (12, 13), dermis formation (14), somitogenesis (4), and junction formation (15, 16). Throughout these processes, the calCAMs appear to be involved in the segregation of differentiating tissues from one another (3, 7).

Consistent with their role in segregation of tissues, three distinct categories of calCAMs have been identified. These include NcalCAM (17–20) and N-cadherin (5, 21) in chicken neural retina; N-CAM (L-CAM) (22–24) in chicken epithelia, E-cadherin (25, 26) and uvomorulin (10) in mouse epithelia, and cell-CAM 120/80 (9) in human epithelia. P-cadherin (6), also in the mouse, is distinct from E- or N-cadherin in its tissue distribution and therefore defines the third category.

Most of the calCAMs were identified by their role in calcium-dependent adhesion of single cells (27, 28). These molecules share other properties as well: they have molecular masses in the range of 120–130 kDa (5, 6, 9, 10, 17, 22, 25); they remain unaltered when intact cells or tissues are trypanized in the presence of calcium (5, 6, 10, 17, 25); and fragments of these molecules, either released into the culture medium or generated by trypsinization of isolated cell membranes in the presence of calcium, have molecular masses in the range of 80–90 kDa (9, 22, 25, 29, 30).

Along with these biochemical characteristics, recent sequencing data indicate that E-cadherin (31) and uvomorulin (32) are identical at the amino acid level and share regions of identity with L-CAM (33, 34). It is also clear that P-cadherin, although similar to E-cadherin, does have distinct domains (35). Shirayoshi et al. (30) have proposed that the cadherins comprise a family of structurally related molecules with tissue-specific domains. Crittenden et al. (20) have also reported data consistent with an extended family of structurally related tissue-specific calCAMs. However, no comparison of the neural and epithelial calCAMs has yet been done either within or between species.

To further clarify these relationships and to extend our immunological studies suggesting that the calCAMs are a family of related yet distinct molecules (20), we have compared NcalCAM and N-cadherin from embryonic chicken neural retina to L-CAM and a newly identified molecule from embryonic chicken liver both immunologically and by two-dimensional peptide mapping.

MATERIALS AND METHODS

Antibodies. NCD-2 is a rat monoclonal antibody to N-cadherin that was kindly provided by M. Takeichi (5). RR2 is a rabbit polyclonal antibody to NcalCAM that recognizes a protein of the same molecular mass as NcalCAM (130 kDa) in every embryonic chicken tissue tested (20).

Mouse monoclonal antibodies to L-CAM were raised against plasma membranes from 14-day embryonic chicken livers. After three injections, splenocytes were fused to mouse NS-1 myeloma cells. Anti-L-CAM is a clone that is liver specific as determined by dot blots of liver and brain membranes. It recognizes a component of ∼124 kDa on blots of one-dimensional NaDodSO₄/PAGE of liver membranes and inhibits calcium-dependent adhesion of embryonic chicken liver cells.

Immunoblots. Samples of intact liver or repaired retina cells (20) were run on two-dimensional gels as described (20) and transferred to Immobilon membranes (Millipore). The papers were blocked for 1 hr at 37°C in 20 mM Tris-HCl, pH 7.4/150 mM NaCl (TBS) containing 5% nonfat dry milk. The primary antibody was incubated with the blot overnight at 4°C in the blocking solution. After several washes in TBS/0.05% Tween, the secondary antibody was incubated with the blot for 2 hr at room temperature followed by washes as described above. Blots incubated with 125I-labeled second

Abbreviations: CAM, cell-adhesion molecule; calCAM, calcium-dependent CAM; L-CAM, liver CAM; N-CAM, neural CAM; CRM-L, cadherin-related molecule in liver.

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antibody (New England Nuclear) were then dried and autoradiographed. Blots incubated with horseradish peroxidase-conjugated second antibody were washed several times in TBS and developed with 4-chloro-1-naphthol (Bio-Rad).

Cell Preparation. Livers were dissected from 10-day embryonic chickens and digested with trypsin (4000 units/ml) in HBSSGK (10 mM Hepes, pH 7.4/150 mM NaCl/glucose (2 mg/ml)/3 mM KCl). After several washes in Tyrode's solution, the tissues were dissociated in HBSSGK containing 1 mM Ca\(^{2+}\) and antipain (10 µg/ml). The cells were plated in BME (Eagle's basal medium; GibCO) containing 2 mg of glucose per ml, 2% (vol/vol) nonessential amino acids (100×; GibCO), 1% glutamine (200 mM; GibCO), and gentamycin, and incubated overnight at 37°C in 10% CO\(_2\)/90% air.

Iodination of Liver Cells. Liver cells prepared as described above were scraped from the dish with a rubber policeman, iodinated by using lactoperoxidase (17), and then trypsinized either in the presence or absence of 5 mM Ca\(^{2+}\) or 5 mM EGTA.

Adhesion Assays. Liver cell monolayers were prepared as described above and incubated overnight in BME containing 2% fetal calf serum. The cells were removed from the dish by trypsin treatment (0.2 mg/ml; 1 min per 100-mm dish, 15 min at 25°C) in the presence of 5 mM Ca\(^{2+}\). For adhesion assays, cells were preincubated with the antibodies for 30 min on ice, pelleted, and resuspended in 0.4 ml of BME containing 0.4 mg of goat anti-mouse or goat anti-rabbit Fab fragments per ml. The cells were then plated into 24-well plates (4 × 10^6 cells per 0.4 ml per well) and rotated (100 rpm, 37°C, 4 hr) in the presence of cycloheximide to inhibit protein synthesis. Where indicated, the cultures contained 10 mM EGTA or immune or preimmune antibodies (60 µg of IgG per ml or 15% serum).

Peptide Mapping. Iodinated spots corresponding to the proteins of interest were excised from two-dimensional gels of iodinated liver or retina cells, extracted from the acrylamide by homogenization, and iodinated to a higher specific activity by using Iodo-Gen (36). Two-dimensional peptide maps were run as described by Elder et al. (37) as modified by Cook et al. (29).

To measure the amount of similarity between the proteins, autoradiographs of the peptide maps were traced onto transparencies. The transparencies were precisely aligned by comparing maps of single proteins to maps of a mixture of two of the proteins. The spots were scored on whether they were unique or shared by two or all three of the molecules. As a negative control, an unrelated protein from another region of a two-dimensional gel was also mapped and analyzed in the same way. A percentage of shared peptides was calculated for each pair of proteins by adding the spots shared by the two and dividing by the total number of spots included in the sum of the two maps, both shared and unique. This number was multiplied by 100 to obtain the percentage of shared peptides. The same type of calculation was used to determine the percentage of peptides shared by three proteins. Each comparison was carried out between two and four times with samples independently prepared. The results were entirely consistent from experiment to experiment. If there was any ambiguity as to the position of a peptide, and therefore whether it was shared, it was considered unique. Thus, the reported percentages are minimal estimates of relatedness.

RESULTS

Antibodies to N-Cadherin Recognize NcalCAM on Two-Dimensional Gels. The two independently identified neural calcium-dependent adhesion molecules (CALM), NcalCAM, and N-cadherin are very similar in their molecular mass, trypsin sensitivity, fragment size, function, and tissue distribution (5, 17, 20). To determine whether the two molecules are the same by immunological criteria, an immunoblot of a two-dimensional gel of iodinated 10-day chicken retina was probed with NCD-2, the monoclonal antibody to N-cadherin. As shown in Fig. 1B, NCD-2 recognized a single component. This component corresponds to the iodinated spot at 130 kDa and pl 4.8 (Fig. 1A) that we have previously identified as NcalCAM (20). Since these molecules share many biochemical properties as well as immunological cross-reactivity, we conclude that NcalCAM and N-cadherin are the same molecule and now refer to both as N-cadherin.

Polyconal Anti-N-Cadherin and Anti-L-CAM Recognize Distinct Components in the Liver. The polyclonal anti-N-Cadherin antisera RR2 recognizes N-cadherin and inhibits calcium-dependent adhesion of embryonic chicken neural retina cells. It also recognizes a 130-kDa molecule in various other embryonic tissues including epithelial, endothelial, and fibroblastic populations (20). Anti-L-CAM antibodies have been reported to recognize a 124-kDa molecule specifically in liver cells and inhibit calcium-dependent adhesion of liver cells (22). To compare the RR2-reactive component to that recognized by anti-L-CAM, the rabbit polyclonal RR2 and a mouse monoclonal anti-L-CAM were used to probe the same immunoblot of a two-dimensional gel of 10-day embryonic chicken liver. Fig. 2B shows the autoradiograph of the RR2-reactive component, while Fig. 2C shows the peroxidase anti-L-CAM-stained component. An autoradiograph of a two-dimensional gel of iodinated liver cells is shown in Fig. 2A for comparison. The molecules recognized by each antiserum are distinct: L-CAM is slightly more basic and has a slightly lower molecular mass than the RR2-reactive component. These results indicate that 10-day embryonic chicken has two molecules related to the calCAMs, L-CAM and a second molecule closely related to N-cadherin, and therefore termed cadherin-related molecule in liver (CRM-L).

Two-Dimensional Peptide Maps Show Strong Similarity Between N-Cadherin and CRM-L. To determine the extent of the relationship between N-cadherin, L-CAM, and CRM-L, two-dimensional peptide maps of complete tryptic digests of all three were prepared. We would expect N-cadherin and CRM-L to be related based on their cross-reactivity with RR2, their similar migration on two-dimensional gels, and their trypsin sensitivity (see Fig. 5). Furthermore, based on function and trypsin stability we would also expect N-cadherin and L-CAM to be related. Even an initial inspection of the peptide maps (Fig. 3) indicates that N-cadherin and CRM-L are very similar in structure. In contrast, similarities between L-CAM and either N-cadherin or CRM-L are not obvious.

Fig. 1. Immunoblot using NCD-2. (A) Autoradiograph of a two-dimensional gel of iodinated chicken retina cells transferred to Immobilon. (B) The same paper as in A after reaction with NCD-2 and horseradish peroxidase-conjugated goat anti-rat IgG. Dotted lines indicate 130 kDa (horizontal) and pl 4.8 (vertical). The point at which these lines cross marks the position of N-cadherin.
To evaluate the relationships between the proteins more quantitatively, detailed comparisons of the number of comigrating peptides in the maps of N-cadherin, L-CAM, CRM-L, and a completely unrelated protein were made. The strong similarity between the peptide maps of the two RR2-reactive components, N-cadherin and CRM-L, is reflected in the high percentage of shared peptides (69%). On the other hand, L-CAM shares only 20% of its peptides with N-cadherin or CRM-L and most of these are shared by all three of the molecules (18%). This is more than three times greater than the percentage of peptides shared by the unrelated protein and any two of the calCAMs (5%). Thus, L-CAM does appear to be related to the cadherins but this relationship is not nearly as strong as the relationship among the cadherins. The peptides shared by N-cadherin, L-CAM, and CRM-L are numbered in Figs. 3 and 4. Fig. 4 is a composite of peptide maps of N-cadherin, L-CAM, and CRM-L. Peptides that are unique to each molecule are shown here as well as the peptides shared by two or all three of the molecules.

Both CRM-L and L-CAM Are Stable to Trypsin in the Presence of Calcium. As N-cadherin remains intact at the cell surface after trypsinization in the presence of calcium (17), it

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**Fig. 2.** Immunoblot using RR2 and anti-L-CAM. (A) Autoradiograph of a two-dimensional gel of iodinated liver cells. (B) Autoradiograph of an immunoblot of a two-dimensional gel of intact liver using RR2 and 125I-labeled goat anti-rabbit IgG. The stained spot at 70 kDa and pl 5.0 is a cytoplasmic component not seen in membrane preparations (20). (C) The same paper as in B after reaction with anti-L-CAM and horseradish peroxidase-conjugated goat anti-mouse IgG. Dotted lines indicate 130 kDa (horizontal) and pl 4.8 (vertical).

**Fig. 3.** Autoradiographs of two-dimensional peptide maps of N-cadherin, CRM-L, L-CAM, and the unrelated protein X. The numbered peptides are shared by N-cadherin, L-CAM, and CRM-L. The peptides marked with an X are shared by N-cadherin and CRM-L and are marked for orientation. HVPE, high voltage paper electrophoresis; chrom, chromatography.
was of interest to ascertain whether CRM-L as well as L-CAM were left intact under the same conditions. Primary cultures of liver cells were surface iodinated (Fig. 5A) and trypsinized in the presence and absence of calcium, and the iodinated proteins were analyzed by two-dimensional NaDodSO₄/PAGE. CRM-L and L-CAM both remain intact although reduced in quantity (as is N-cadherin) after trypsin treatment in the presence of calcium (Fig. 5B). Both spots are eliminated by trypsinization in the absence of calcium (Fig. 5C).

Anti-L-CAM But Not Polyclonal Anti-N-Cadherin Inhibits Calcium-Dependent Adhesion of Liver Cells. To determine whether CRM-L plays a role in calcium-dependent adhesion of liver cells, the effects of RR2 and anti-L-CAM were compared. Liver cells with an intact functional calcium-dependent cell-adhesive system on their surface are able to form clusters in the presence of cycloheximide only when calcium is present (Fig. 6A). Although RR2 inhibits calcium-dependent adhesion of neural retina cells and recognizes CRM-L, it had no effect on the calcium-dependent adhesion of liver cells (Fig. 6C). On the other hand, anti-L-CAM is an effective inhibitor (Fig. 6D), inhibiting calcium-dependent adhesion to the same extent as the absence of calcium (Fig. 6B). RR2 was also tested for an effect on calcium-dependent adhesion in the presence of a half-maximal inhibitory dose of anti-L-CAM. No additive effect of RR2 was seen (data not shown).

DISCUSSION

The data presented here clarify the structural relationships between three different molecules with properties characteristic of calcium-dependent CAMs in the embryonic chicken. One of these molecules, previously referred to as NcadCAM by our laboratory (18-20), is shown to be identical to N-cadherin studied by Takeichi’s group (5, 21) and is hereafter referred to as N-cadherin.

The other two molecules were both detected in chicken liver. One was identified on the basis of its immunological cross-reactivity with antisera raised against N-cadherin. This molecule shares a number of characteristics with N-cadherin and we therefore refer to it as CRM-L, for cadherin-related molecule in liver. In addition to being the same molecular mass and pl, it is also protected from trypsinization by calcium and has a very similar two-dimensional peptide map, sharing more than two-thirds of its peptides with N-cadherin. It is important to note that N-cadherin and CRM-L have some peptides that are unique to each, consistent with the fact that they have immunologically distinct domains (20). Whether

![Image of a composite line drawing of the maps of N-cadherin, CRM-L, and L-CAM. Numbered peptides correspond to those in Fig. 3.](image)

![Image of effects of RR2 and anti-L-CAM on calcium-dependent adhesion of liver cells.](image)

![Image of effects of trypsinization in the presence of calcium on CRM-L and L-CAM.](image)
such domains are a result of transcription of different genes or transcriptional, translational, or posttranslational modifications of the same gene product is not known. Regardless of the mechanism by which these domains are created, because of their tissue specificity, they may be relevant to the ability of embryonic cells to discriminate in the formation of adhesions.

The second molecule in chicken liver, L-CAM, shares with the cadherins many characteristics, including its surface localization and stability to trypsinization. However, in terms of molecular mass, pl, and peptide homologies, this molecule is clearly less closely related to either N-cadherin or CRM-L than the latter two are to each other. The lower degree of peptide similarity between L-CAM and N-cadherin is consistent with the report that chicken L-CAM cDNA probes do not hybridize with mRNA from chicken brain (33, 34). Nevertheless, the degree of peptide similarity between L-CAM and the cadherins is significantly greater than that between L-CAM and an unrelated protein and is likely to represent a substantial similarity in primary sequence. This is supported by the fact that membrane proteins with dissimilar peptide maps have been reported to share extensive primary sequence 

In chicken liver tissue, L-CAM appears to be present in greater amounts than CRM-L, as evidenced by the amount of iodinatable material present in intact cells. This apparent predominance could explain the fact that antibodies against L-CAM completely block liver cell adhesion, whereas antibodies against N-cadherin, which cross-react with CRM-L, do not. It is also possible that the antisera we are using to block CRM-L function does not recognize a functional epitope. Because CRM-L shows such strong homology with N-cadherin, it seems reasonable to expect that it will play a role in adhesive interactions during morphogenesis that are not detected by these in vitro cell-adhesion assays.

The present work also raises the question of whether murine E-cadherin is more closely related to N-cadherin or L-CAM. It is interesting to note that the E-cadherin derived from mouse liver tissue differs in molecular mass from the E-cadherin derived from mouse embryonal carcinoma cells (28), suggesting that the term E-cadherin may include more than one type of calCAM in the mouse. Our studies suggest that the calCAMs may represent a more diverse group of molecules than previously suspected, including multiple structural and functional types present in the same tissue. This diversity could perhaps be important in more subtle tissue interactions during morphogenesis. Our results also emphasize the importance of using inter- and intraspecies comparisons in future studies designed to assess similarities and differences among the calCAMs.

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