

Molecular Organization of the Uvomorulin–Catenin Complex

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Abstract. The Ca^{2+} -dependent cell adhesion molecule uvomorulin is a member of the cadherin gene family. Its cytoplasmic region complexes with structurally defined proteins termed α -, β -, and γ -catenins. Here we show that A-CAM (N-cadherin), another member of this gene family, also associates with catenins suggesting that this complex formation may be a general property of the cadherins. For uvomorulin it has been found that this association with catenins is of crucial importance for the adhesive function, but little is known about the molecular organization of the uvomorulin–

catenin complex. Using a combination of biochemical analyses we show that a single complex is composed of one molecule of uvomorulin, one or two molecules of β -catenin, and one molecule of α -catenin. Furthermore, β -catenin seems to interact more directly with uvomorulin. In pulse–chase experiments β -catenin is already associated with the 135-kD uvomorulin precursor molecule but the assembly of the newly synthesized α -catenin into the complex is only detected around the time of endoproteolytic processing.

UVOMORULIN belongs to the class of Ca^{2+} -dependent cell adhesion molecules (CAMs).¹ Although it was first described to be involved in the compaction process of mouse preimplantation embryos (Kemler et al., 1977; Hyafil et al., 1981), the present notion is that its major biological role seems to be related to the generation and maintenance of epithelial cell layers (for review see Kemler et al., 1989; Kemler et al., 1990), not only during development (Vestweber and Kemler, 1985), but also in adult tissues (Behrens et al., 1985; Gumbiner et al., 1988). The molecular cloning of uvomorulin/E-cadherin (Nagafuchi et al., 1987; Ringwald et al., 1987) and the comparison of its primary structure with those of other Ca^{2+} -dependent CAMs (Gallin et al., 1987; Nose et al., 1987; Hatta et al., 1988) revealed that these proteins constitute a family of functionally related molecules also termed cadherins (for review see Takeichi, 1988; Kemler et al., 1989). More recently additional members of this gene family have been described including mouse muscle M-cadherin (Donalies et al., 1991) and some of the desmosomal proteins (Koch et al., 1990; Wheeler et al., 1991; Mechanic et al., 1991).

Uvomorulin is a transmembrane glycoprotein of 120 kD with a cytoplasmic region of ~150 amino acid residues. Recently, we have shown that uvomorulin is noncovalently associated by its cytoplasmic domain with three proteins of 102, 88, and 80 kD termed catenin α , β , and γ , respectively (Ozawa et al., 1989). This association is controlled by a specific domain in the carboxy-terminal 72-amino acid

residues (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990). Since the amino acid sequence of the catenin-binding domain of uvomorulin exhibits a particularly high degree of homology with the corresponding sequences of other cadherins (86–92% identity), it was proposed that catenins might also associate with other members of this gene family (Ozawa et al., 1989). Such a view is supported by recent studies on α -catenin (Nagafuchi et al., 1991; Herrenknecht et al., 1991). For uvomorulin it has been shown that the complex formation influences the extracellular mediated adhesive function and is involved in the connection of uvomorulin to the cytoskeletal network (Ozawa et al., 1990). Despite the functional importance of the complex formation little is known about its structure and assembly. Here we characterize the complex in more detail.

Materials and Methods

Cells

The following transfected mouse L-tk⁻ cells used in this study have been described before (Ozawa et al., 1989, 1990; Ozawa and Kemler, 1990): L1-1 cells express wild-type uvomorulin, LΔC10 cells express a mutant uvomorulin polypeptide lacking the cytoplasmic 105 amino acid residues, and LF-7 cells express the uvomorulin precursor polypeptide in which the recognition site for endoproteolytic enzymes was replaced by a recognition site for the highly specific serum coagulation factor Xa. C1-4 is a transfected chicken embryo fibroblast cell line expressing mouse uvomorulin. Primary cultures of 14-d-old chicken embryos were kind gifts of Dr. A. Dierich (University of Strasbourg, Strasbourg, France). Chicken embryo liver cells were isolated from 14-d-old embryos using 0.01% trypsin and 1 mM EDTA in PBS. To obtain transfectants expressing both normal uvomorulin and the precursor mutant polypeptide, the pSUMPF vector which encodes the mutant uvomorulin (Ozawa and Kemler, 1990) was introduced into L1-1 cells, which express normal uvomorulin. Since L1-1 cells already contained the neomycin gene, another plasmid for selection (pStk)

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1. *Abbreviations used in this paper:* CAM, cell adhesion molecule; DTSSP, dithiobis (sulfosuccinimidyl) propionate.

was constructed by subcloning a 1.8-kb BglII-BamHI fragment of herpes simplex virus thymidine kinase gene into the BglII-BamHI site of pSV2tkneo β (Nicolas and Berg, 1983). After transfection by the calcium-phosphate method, cells were selected with HAT medium (hypoxanthine, aminopterin, and thymidine) and transfectants expressing the mutant polypeptide were isolated by fluorescence-activated cell sorter analysis with antiprecursor antibodies (Ozawa and Kemler, 1990).

Antibodies

Antibodies against the extracellular part of mature uvomorulin (anti-gp84) were affinity purified as described (Vestweber and Kemler, 1985). Preparation and purification of antibodies against the precursor segment of uvomorulin (antiprecursor) have been described (Ozawa and Kemler, 1990). Mouse monoclonal antibodies against A-CAM, ID-7.2.3 (Volk and Geiger, 1984), and FA-5 (Volk et al., 1990) were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit antibody against chicken L-CAM (Gallin et al., 1987) was a kind gift of Dr. B. A. Cunningham (The Rockefeller University, New York).

Immunoblot and Immunoprecipitation

Immunoblotting was carried out as described (Ozawa et al., 1989). Cells (1×10^6) were labeled with 50 μ Ci/ml of [35 S]methionine (Amersham Corp., Arlington Heights, IL) in DME without methionine, 10% FCS for 16 h, and solubilized with PBS containing 1% Triton X-100, 1% NP-40, 2 mM CaCl₂, and 1 mM PMSF. After centrifugation supernatants were incubated with affinity-purified antibodies and immunocomplexes were collected by protein A-Sepharose CL 4B (Pharmacia Fine Chemicals, Piscataway, NJ) as described (Vestweber and Kemler, 1985). In the case of monoclonal antibody FA-5 against A-CAM, protein A-Sepharose was precoated with affinity-purified rabbit antibodies against mouse IgG (Jackson ImmunoResearch Lab., Inc., West Grove, PA).

Peptide pattern analyses of the coprecipitated proteins were carried out according to Ozawa et al. (1989). To analyze the stability of the uvomorulin-catenin complex, immunocomplexes were incubated with 1 ml of the solutions listed in Table I for 30 min on ice. After centrifugation the immunocomplexes were boiled with SDS-PAGE sample buffer for 3 min and analyzed by SDS-PAGE. Fluorography was performed on Enlightening (New England Nuclear, Boston, MA) impregnated gels using Kodak XAR-5 film. Fluorographs were scanned and the integrated optical density of bands was determined using a laser densitometer (Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD).

Immunoaffinity Chromatography

Protein A-Sepharose CL 4B (0.6 ml bed vol) was coupled with affinity-purified anti-gp84 antibodies (1 mg) in 5 ml of 0.1 M sodium borate buffer, pH 8.0, containing 5 mg/ml of dimethylsuberimidate for 30 min at room temperature, washed with 0.1 M potassium phosphate buffer (pH 7.0), and with 3 ml of 0.1 M glycine-HCl buffer (pH 2.5). Embryonal carcinoma F9 cell tumors (1 g) collected from syngenic 129 SV mice were homogenized in TBS containing 2 mM CaCl₂, 1 mM PMSF, and 1% NP-40 and centrifuged at 100,000 *g* for 30 min. The supernatants were applied to the anti-gp84 column washed with TBS containing 2 mM CaCl₂, 1 mM PMSF, 0.1% NP-40, and bound material was eluted with 0.1 M glycine-HCl buffer (pH 2.5), 2 mM CaCl₂, 0.1% NP-40. The eluates were precipitated by the addition of 4 vol of ethanol, boiled in SDS-PAGE sample buffer, and subjected to SDS-PAGE. After staining with Coomassie blue, different amounts of the eluates were scanned and the integrated optical density of bands was determined as above.

Chemical Cross-linking and Two-dimensional (Nonreducing-Reducing) SDS-PAGE

Cells (1×10^6) labeled with [35 S]methionine as described above were lysed with 500 μ l of PBS containing 2 mM CaCl₂, 1 mM PMSF, and 40 mM octylglucoside or 1% Triton X-100. After centrifugation, aliquots (225 μ l) of the supernatants were mixed with 25 μ l of the cross-linking reagent dithiobis (sulfosuccinimidyl) propionate (DTSSP) in PBS. The mixtures were incubated for 30 min at 4°C, treated with 100 μ l of 0.1 M glycine-HCl (pH 10.0), for 30 min at 4°C, and after adding 10 μ l of 0.75 M Tris-HCl (pH 8.8), subjected to immunoprecipitation. The immunocomplexes were boiled for 3 min in SDS-PAGE sample buffer without reducing reagents and subjected to SDS-PAGE on 4% acrylamide gels (first dimension). After

electrophoresis, gels were incubated with SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 1 h at room temperature and subjected to electrophoresis on 8% acrylamide gels (second dimension).

Sedimentation Analysis

Cells (1×10^6) were labeled with [35 S]methionine as described above and lysed with TBS (150 mM NaCl, 20 mM Tris HCl, pH 7.4) containing 2 mM CaCl₂, 1 mM PMSF, and 1% Triton X-100. After centrifugation, supernatants (250 μ l) were layered onto 3.8-ml linear 5–20% (wt/wt) sucrose gradients prepared in TBS containing 2 mM CaCl and 0.1% Triton X-100, and centrifuged 149,000 *g* for 16 h in the AH-650 rotor (Sorvall Instruments, Du Pont, Germany) at 4°C. Gradients were fractionated from bottom to top into 12 fractions and the fractions were analyzed by immunoprecipitation and SDS-PAGE. The following protein standards of known S values were centrifuged on replicate gradients: catalase, 11.4 S; aldolase, 7.4 S; BSA, 4.6 S. Their distributions in the sucrose gradients were controlled by SDS-PAGE.

Pulse-Chase Experiments

Cells (1×10^6) were preincubated for 1 h in methionine-free MEM supplemented with 10% FCS and incubated for 7 min in the same medium with 250 μ Ci/ml of [35 S]methionine. Cells were washed with MEM supplemented with 10% FCS and 2 mM cold methionine and incubated in this medium (zero time of chase). Cells were solubilized at the times indicated in the figure and subjected to immunoprecipitation.

Results

Association of Chicken A-CAM (N-cadherin) with Catenins

Primary chicken embryonic fibroblasts, which express A-CAM (N-cadherin, Volk and Geiger, 1984), and a transfected chicken embryo fibroblast cell line (C1-4) expressing mouse uvomorulin (Ozawa et al., 1989), were compared in immunoblot and immunoprecipitation analysis. In immunoblots both CAMs were detected by the respective antibodies (Fig. 1 A, lanes 1 and 3). Upon immunoprecipitation anti-A-CAM antibodies coprecipitated three proteins of 102, 88, and 80 kD (Fig. 1 A, lane 4). These proteins were identical to those of chicken α -, β -, and γ -catenin detected by anti-uvomorulin antibodies from C1-4 cells as the respective

Table I. Stability of the Uvomorulin-Catenin Complex

Treatment	Catenins retained		
	Relative ratios to control		
	α	β	γ
Control*	1.0	1.0	1.0
5 mM Tris-HCl (pH 8.0) and 1 mM EDTA	1.00	0.97	1.02
3 M NaCl	0.62	0.85	0.35
0.6 M KI	0.39	0.56	0
2% Triton X-100	1.02	1.02	1.02
2% Triton X-100 and 1% SDS	0	0	0
2% Triton X-100 and 1% Deoxycholate	0.96	1.01	0.98
40 mM Octylglucoside	0.13	0.98	0.91
0.1 M β -Methylglucoside	1.01	0.96	1.02

Uvomorulin-catenin complex was collected with anti-gp84 and protein A-Sepharose, washed, and incubated with solutions listed above for 30 min at 4°C (see Materials and Methods). All reagents were dissolved in PBS unless otherwise specified.

* Control; immunoprecipitation washing buffer, 50 mM Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl, 0.05% NP-40, and 0.1% ovalbumin.

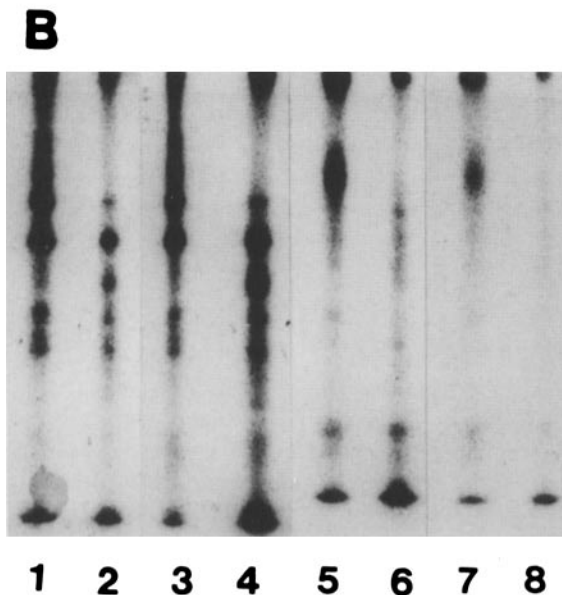
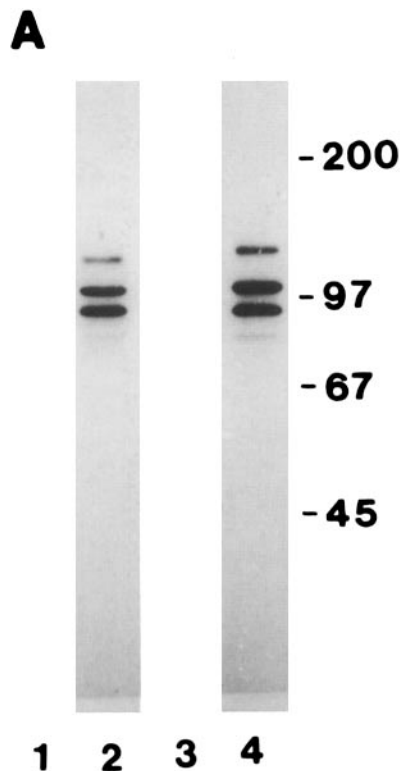


Figure 1. Characterization of A-CAM-associated proteins. (A) Immunoprecipitation from cell lysates of transfected chicken embryo fibroblast cell line, C1-4, (lane 1 and 2) and chicken embryo fibroblasts (lane 3 and 4) with anti-uvomorulin (lane 2) and anti-A-CAM antibodies (lane 4); lanes 1 and 3 = controls. (B) Peptide map analysis of the associated proteins. α -catenin (lanes 1 and 2) and β -catenin (lanes 5 and 6) from C1-4 cells or the 102-kD (lanes 3 and 4) and the 88-kD (lanes 7 and 8) A-CAM-associated proteins were subjected to Cleveland digestion with 2 ng (lanes 1, 3, 5, and 7) or 20 ng (lanes 2, 4, 6, and 8) of *Staphylococcus aureus* V8 protease.

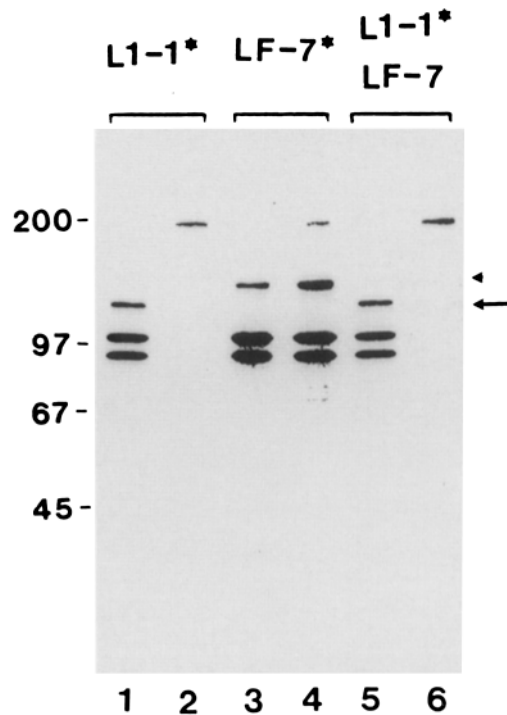


Figure 2. Individual components of the complex cannot reassociate during solubilization. L cells expressing wild-type (L1-1) or mutant uvomorulin (LF-7) were labeled with [35 S]methionine. Cell lysates prepared from labeled L1-1 cells (L1-1*) or labeled LF-7 cells (LF-7*) or the mixture of the same number of labeled L1-1 cells and unlabeled LF-7 cells (L1-1*-LF-7) were subjected to immunoprecipitation with antibodies against the extracellular part of mature uvomorulin (anti-gp84, lanes 1, 3, and 5) or antibodies against the precursor segment (antiprecursor, lanes 2, 4, and 6). Immunoprecipitates were analyzed by 8% PAGE. The arrowhead indicates the position of the precursor, and the arrow the position of mature uvomorulin.

peptide pattern comparison revealed (Fig. 1 B). Similar experiments were performed with rabbit anti-chicken L-CAM antibodies and metabolically labeled 13-d-old chicken embryonic liver cells. It was found that chicken L-CAM complexes with three proteins with molecular weights identical to catenins although the amount of immunoprecipitated material was insufficient for unambiguous peptide pattern analysis (not shown). These results indicate that catenins can complex with several cadherins, which is in good agreement with recent results obtained with anti- α -catenin antibodies (Herrenknecht et al., 1991).

Stability of Uvomorulin-Catenin Complex

The uvomorulin-catenin complex is detected after solubilization of cells with Triton X-100 or NP-40. To test whether some components of the complex can be exchanged or reassociate after solubilization, mixing experiments were performed between cell lysates of transfectants expressing either normal uvomorulin (L1-1) or a precursor mutant protein (LF-7). The mutant protein can be detected by specific antiprecursor antibodies (see Materials and Methods; and Ozawa and Kemler, 1990). The specificity of antiprecursor and anti-gp84 antibodies is shown in Fig. 2, lanes 1-4, by comparing L1-1 and LF-7 cells in immunoprecipitation analysis.

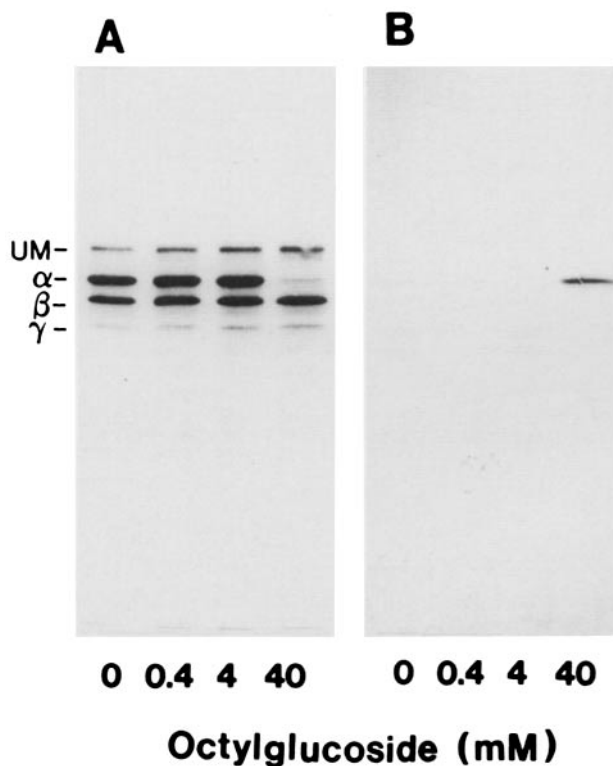


Figure 3. Selective dissociation of α -catenin from the complex by octylglucoside. Uvomorulin-catenin complex was collected from triton X-100 L1-1 cell lysates, and incubated in octylglucoside/PBS as indicated. The complexed material (A) and released protein (B) were separated and analyzed by SDS-PAGE. In B only one third of the total material was put on the gel and the rest was used for peptide analysis to verify the identity with α -catenin (not shown).

Antiprecursor antibodies detect only the precursor mutant which also complexes with catenins. When [³⁵S]methionine-labeled L1-1 cells were mixed with nonlabeled LF-7 cells and solubilized, labeled catenins were exclusively detected with anti-gp84 antibodies (lanes 5 and 6). Therefore, individual components of the complex seem not to exchange or to re-associate during solubilization. The result also shows that the uvomorulin-catenin complex is efficiently formed only if uvomorulin and catenins are produced by the same cell.

The stability of the complex was examined by treating immune complexes with a variety of solubilization conditions. As shown in Table I, the uvomorulin-catenin complex is rather stable. SDS was the only reagent that dissociated catenins completely from uvomorulin. EDTA had no effect indicating that divalent cations are not necessary for the association. Treatment with a high concentration of NaCl or KI resulted in partial removal of α - and γ -catenin. Although the complex was stable in Triton X-100 or in a combination of Triton X-100 and deoxycholate, another nonionic detergent, octylglucoside (40 mM), reduced the amount of α -catenin in the complex significantly (Fig. 3 A). The α -catenin component was released intact from the complex as judged by the electrophoretic mobility (Fig. 3 B). β -Methylglucopyranoside had no effect showing that the sugar portion is not responsible for the action of octylglucoside.

These results gave the first indication that β -catenin might interact directly with uvomorulin since it was found associated with uvomorulin in the absence of α - or γ -catenin.

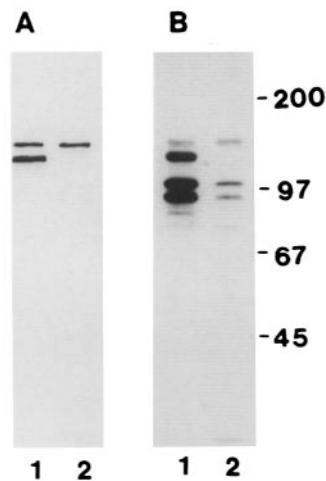


Figure 4. Immunoblot and immunoprecipitation analysis of L cells expressing wild-type uvomorulin and the mutant precursor protein. Cells were subjected to immunoblot (A) or immunoprecipitation (B) analysis with anti-gp84 (lanes 1) or antiprecursor (lanes 2) antibodies as described in Materials and Methods. As seen in B, lane 2, these two uvomorulin polypeptides cannot be found together in a single complex.

One Uvomorulin in a Single Complex

To examine whether uvomorulin can form homodimers or whether catenins can connect several uvomorulin molecules, double transfectants were isolated expressing both normal uvomorulin and the mutant precursor polypeptide. Since the mutant protein has the ability to complex with catenins, immunoprecipitations with antiprecursor antibodies should in addition reveal normal uvomorulin if both proteins are associated in some way. Western blot analysis with anti-gp84 and antiprecursor antibodies revealed that transfectants express both normal (120 kD) and the 135-kD mutant polypeptides (Fig. 4 A). Upon immunoprecipitation analysis of [³⁵S]methionine-labeled cells, anti-gp84 antibodies precipitated as expected normal uvomorulin and the mutant uvomorulin together with catenins (Fig. 4 B, lane 1). The amount of mutant uvomorulin expressed on these cells was less than one fifth of normal uvomorulin. Antiprecursor antibodies, however, precipitated only the mutant polypeptides together with catenins (Fig. 4 B, lane 2). The results exclude the possibility that the precursor and mature proteins are incorporated into a single complex. These results do not rule out the possibility that mature proteins can form multimers in situ.

Sedimentation Analysis of the Complex

To examine the extent of the complex formation of uvomorulin and to estimate the overall size of uvomorulin-catenin complex, sedimentation analyses were performed. L1-1 cells expressing normal uvomorulin were labeled with [³⁵S]methionine and solubilized with Triton X-100. The lysate was subjected to centrifugation on 5–20% (wt/wt) sucrose gradients, and the gradient fractions were analyzed by immunoprecipitation with anti-gp84 antibodies and SDS-PAGE.

As shown in Fig. 5 A, uvomorulin sediments as a single species at about 7.4 S together with α - and β -catenin demonstrating that almost all of uvomorulin is associated with catenins with virtually no free uvomorulin. The sedimentation analysis revealed that the complex sedimented in the range comparable to the marker protein, aldolase, which has a molecular mass of 160 kD. Thus the molecular mass of the complex obtained by this analysis is rather small compared to the calculated molecular mass of 310 kD which is calculated on the assumption that the complex contains only one molecule of uvomorulin, α -, and β -catenin. Since γ -catenin was not

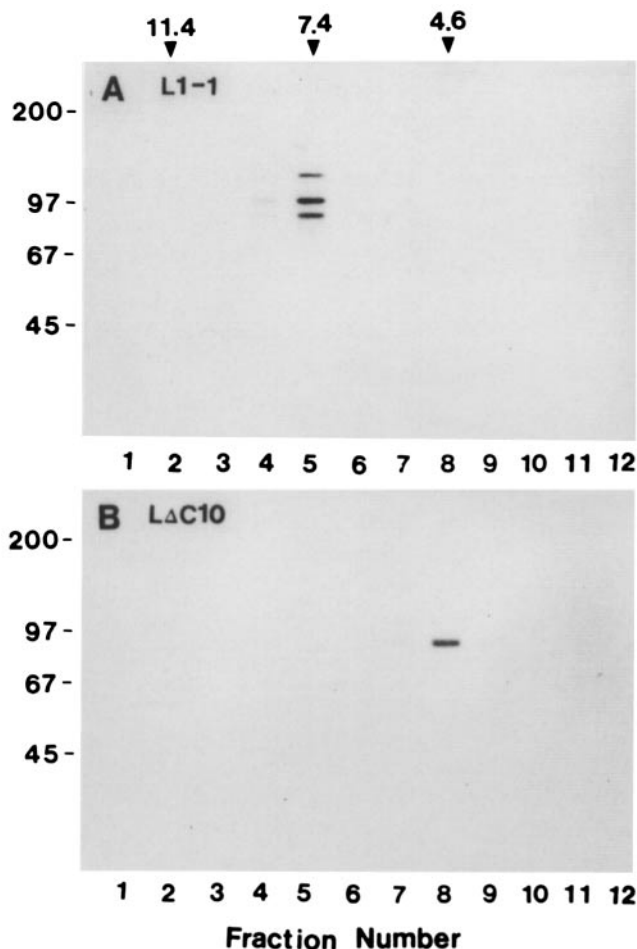


Figure 5. Sedimentation analysis of uvomorulin-catenin complex on sucrose gradients. L cells expressing wild-type uvomorulin (L1-1, *A*) or mutant uvomorulin which cannot associate with catenins (LΔC10, *B*) were labeled with [³⁵S]methionine. Cell lysates were subjected to sucrose gradient centrifugation as described in Materials and Methods. 12 fractions were collected and numbered consecutively from the bottom to the top of the tubes. Each fraction was immunoprecipitated with anti-gp84 antibodies and analyzed by SDS-PAGE. The peak distribution of protein standards of known S values are shown in the top of the figure.

detected, this component seems to become dissociated from the complex during centrifugation. When LΔC10 cells expressing mutant uvomorulin polypeptide which cannot associate with catenins were subjected to the same analysis, the mutant polypeptides sedimented as a single species at 4.6 S (Fig. 5 *B*).

Determination of the Molar Ratios of Components

The stoichiometry of α -catenin to uvomorulin in the complex was determined by densitometry obtained from 12 independent immunoprecipitation experiments. Immunoprecipitates were collected from L1-1 cells labeled with [³⁵S]methionine for >16 h (in these cells, apparent half-lives of uvomorulin and catenins were ~2 h; data not shown). The amount of each component was compared by densitometry of the fluorographs (Table II). The recent cloning of α -catenin (Herrenknecht et al., 1991; Nagafuchi et al., 1991) revealed that the primary translation product contains 28

Table II. Relative Peak Area of the Components in the Complex

	[³⁵ S]Methionine*	Coomassie blue†
Uvomorulin	1.0	1.0
α -Catenin	2.9	4.0
β -Catenin	1.5	1.4
γ -Catenin	0.5	0.3

* Uvomorulin-catenin complex was immunoprecipitated from L1-1 cells labeled with [³⁵S]methionine and subjected to SDS-PAGE as described in Materials and Methods. The peak area of each component was determined by densitometry of fluorographs and expressed as a relative amount to uvomorulin. The average of 12 experiments is presented.

† Uvomorulin-catenin complex was purified from F9 cell tumors by immunoaffinity chromatography on anti-gp84-Sepharose as described in Materials and Methods, subjected to SDS-PAGE, and stained with Coomassie blue. The peak area of each component was determined by densitometry of Coomassie blue-stained gels and expressed as a relative amount to uvomorulin. The average of three experiments is presented.

methionine residues, which is rather high compared to the 10 methionines of uvomorulin (Ringwald et al., 1987). The relative peak area of α -catenin to uvomorulin was found to be 2.9 (Table II), which strongly suggests 1:1 molar ratio of α -catenin and uvomorulin. Similar comparison for β - and γ -catenin was not possible because the respective primary structures are presently not known. The approximate ratio of these catenins to uvomorulin was determined by densitometry of Coomassie blue-stained gels (Table II). This analysis suggests that there are approximately one (or two) molecule(s) of β -catenin and one (or less) molecule of γ -catenin per molecule of uvomorulin in the complex. The integrated optical density of each band may not reflect the actual amount of each polypeptide because of differences in the avidity of the dye to each polypeptide. This may be the reason for differences in the determination of the relative peak area of α -catenin obtained from metabolically labeled or Coomassie blue-stained material.

Nearest Neighbor Analysis within the Complex

The spatial relationship of uvomorulin and catenins in the complex was analyzed using the homo-bi-functional cross-linking reagent DTSSP and two-dimensional SDS-PAGE analysis under reducing or nonreducing conditions. DTSSP reacts covalently with amino groups and its span of 12 Å requires a close special relationship between proteins to be cross-linked. The internal disulfide bond of DTSSP allows cleavage with reducing agents.

In the first series of experiments, immunoprecipitates were analyzed from L1-1 cells solubilized with 40 mM octylglucoside where α -catenin is absent (Fig. 3). Control LΔC10 cells expressing mutant uvomorulin which cannot associate with catenins were included. In the first dimension, a cross-linked complex from L1-1 cells migrated as an indistinct band of 180–220 kD, centering at 200 kD (Fig. 6 *A*) which after reduction was cleaved into uvomorulin and β -catenin of 120 and 88 kD, respectively (Fig. 6 *B*). The cross-linked product has a molecular mass of ~200 kD which is in good agreement with the predicted molecular mass of the complex with one uvomorulin and one β -catenin (208 kD), although the possibility of a mixture between uvomorulin dimers and uvomorulin/ β -catenin cannot be completely ruled out.

In another series of experiments, cells were solubilized with Triton X-100/NP-40, lysates were treated with DTSSP and immunoprecipitated. Under nonreducing conditions,

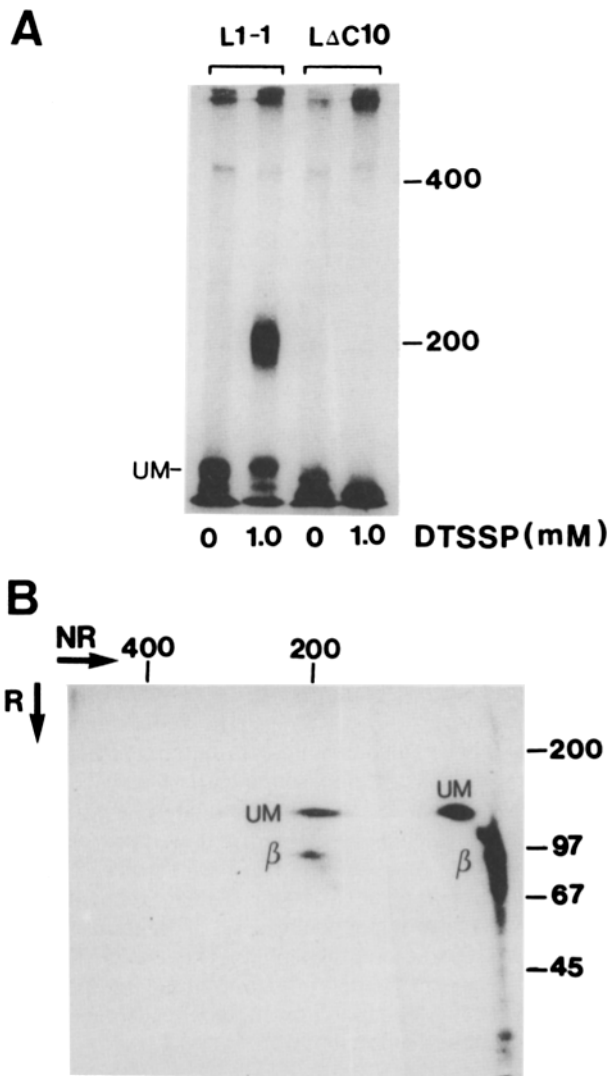


Figure 6. Chemical cross-linking and two-dimensional (nonreducing-reducing) SDS-PAGE analysis of uvomorulin-catenin complex solubilized with 40 mM octylglucoside. Cell lysates of L1-1 or L Δ C10 cells were cross-linked with DTSSP, immunoprecipitated with anti-gp84 antibodies, and electrophoresed on 4% first dimension gel (nonreducing, *A*). Part of the cross-linked complex from L1-1 cells was further analyzed on second dimension 8% gel (reducing, *B*).

three cross-linked products were detected (Fig. 7 *A*). The highest molecular mass product migrated at 310 kD (210–360 kD). The other two products migrated together as a broad band around 155–200 kD. Analysis of the cross-linked products on the second reducing gel revealed that the 310-kD product is composed of uvomorulin and α - and β -catenin (Fig. 7 *B*).

The minor product of \sim 200 kD gave only faint bands most likely composed of uvomorulin and α -catenin (Fig. 7 *B*). The other product centering at 175 kD seems to contain α -catenin- β -catenin heterodimers, which could have been precipitated because of noncovalent association with uvomorulin (Fig. 7 *B*). The relative peak areas of the different components varied in the different cross-linked products and in complexes collected by normal immunoprecipitations. For example, from the densitometry of the fluorographs the 310-

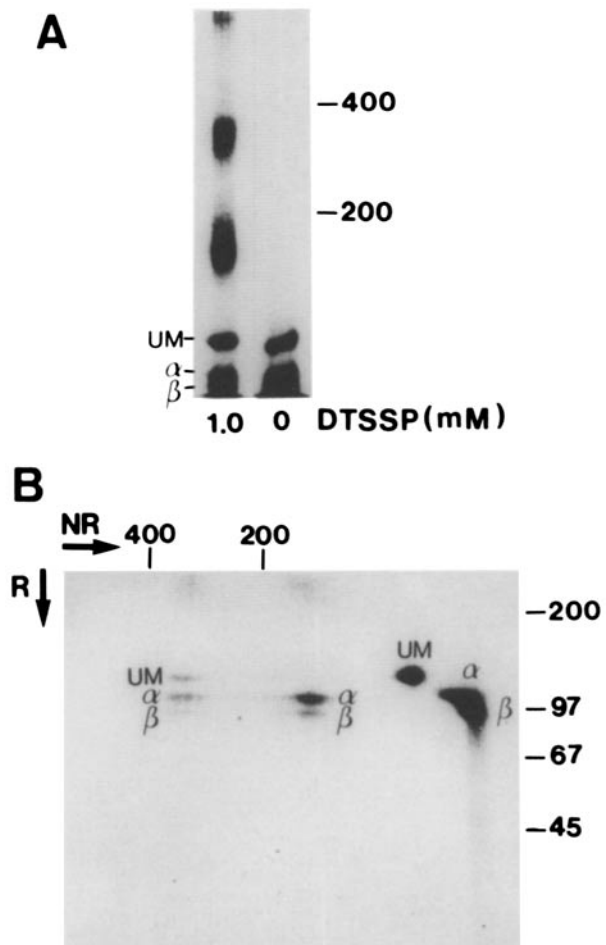


Figure 7. Chemical cross-linking and two-dimensional (nonreducing-reducing) SDS-PAGE of the uvomorulin-catenin complex solubilized with Triton X-100/NP-40. Cell lysates of L1-1 cells after cross-linking, with DTSSP were immunoprecipitated with anti-gp84 antibodies and subjected to electrophoresis on 4% nonreducing gel (*A*). Part of the cross-linked material from *A* was reduced and analyzed on an 8% second-dimension gel (*B*).

kD product could also contain uvomorulin $_2$ - α -catenin and/or uvomorulin $_2$ - β -catenin. In addition, the formation of α -catenin- β -catenin cross-linked products revealed a close spacing of these components in the complex. Nevertheless these cross-linking experiments support two major conclusions. First, apart from uvomorulin and catenins there seems to be no additional protein(s) in close spatial contact within the complex. Second, since the largest cross-linked product obtained was of 310 kD, the complex seems to be composed of one molecule of uvomorulin, α -, and β -catenin. Under the conditions used γ -catenin was not detected in the cross-linked product, which does not, however, exclude the possible direct association of γ -catenin to uvomorulin.

Assembly of the Uvomorulin-Catenin Complex

The assembly of uvomorulin-catenin complex was examined by pulse-chase experiments. L1-1 cells were pulse labeled with [35 S]methionine for 7 min, and chased with excess of unlabeled methionine for different times as indicated in Fig. 8. Cell lysates were subjected to immunoprecipitation with anti-gp84 antibodies and the complexes were analyzed by

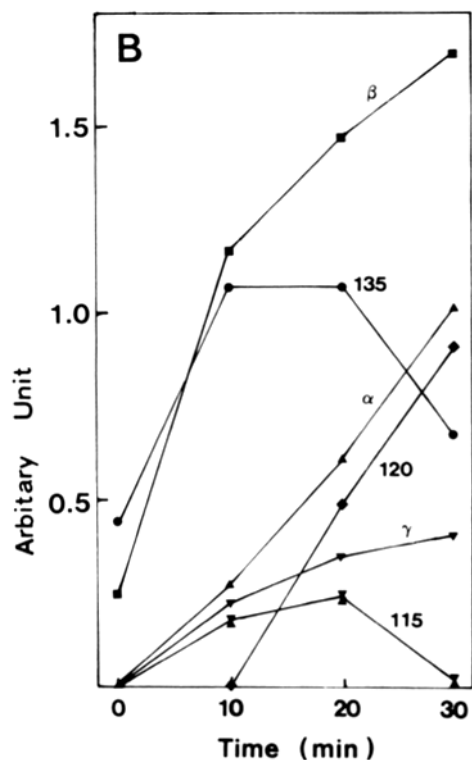
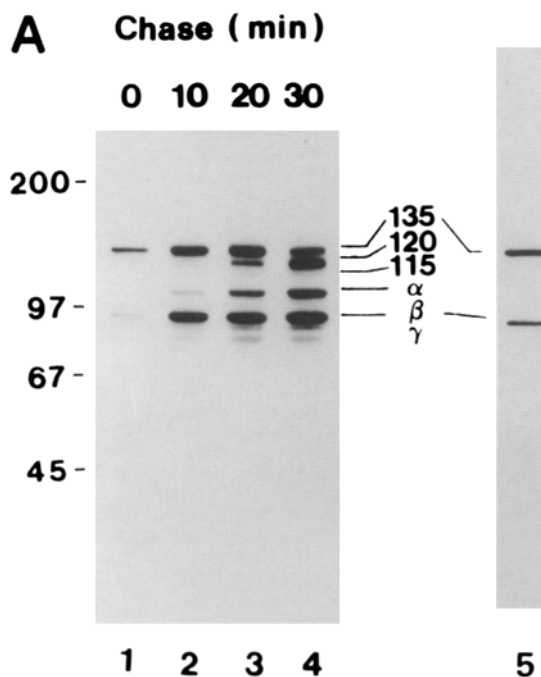


Figure 8. Assembly of uvomorulin-catenin complex. (A) L1-1 cells were pulse-labeled for 7 min and chased for 0, 10, 20, or 30 min as indicated. Cells were lysed and subjected to immunoprecipitation with anti-gp84 antibodies. (B) Graphical representation of A. The integral optical density of bands was determined as described in Materials and Methods.

SDS-PAGE. Antibodies reacting specifically with the precursor polypeptide of uvomorulin were included as a control.

At time 0 the 135-kD precursor of uvomorulin was already associated with β -catenin as shown with anti-gp84 (Fig. 8 A, lane 1) and antiprecursor antibodies (Fig. 8 A, lane 5). After 10 min of chase, when uvomorulin was still in its 135-kD precursor form but a small amount of nonglycosylated 115-kD uvomorulin was already detectable, significant amounts of β -catenin and small amounts of α -catenin were found in the complex (Fig. 8 A, lane 2, and 8 B). The mature uvomorulin of 120 kD appeared after 20 min and its amount increased thereafter (Fig. 8 A, lanes 3 and 4).

These results indicate that β -catenin associates with uvomorulin immediately after synthesis and that the association of α -catenin may correlate with the time of endoproteolytic processing.

Discussion

In the present study we have shown that the complex formation with catenins is most likely a general feature of members of the cadherin gene family. For uvomorulin it has been demonstrated that this association controls the cell adhesion function and the connection of uvomorulin with microfilaments (Ozawa et al., 1990). Therefore, it is quite possible that the association with catenins is also required for the function of other cadherins (Nagafuchi and Takeichi, 1989; Wheelock and Knudsen, 1991). We used various biochemical methods to characterize the molecular organization of the complex. The complex is composed of one molecule of uvomorulin, one molecule of α -catenin, and one or two molecules of β -catenin. γ -Catenin seems to be rather loosely associated since it cannot be detected in all analytical methods used. Although we could not definitively determine the stoichiometry of the components, the minimum molecular mass of the complex seems to be 390 kD (120 + 102 + 88 + 80).

The sedimentation analysis by sucrose gradients after Triton X-100 solubilization revealed that almost all uvomorulin is associated with catenins. The sedimentation characteristic (~ 7.4 S) of the complex suggests that it has an elongated shape in solution since the calculated minimum molecular mass differs from the sedimentation characteristics. One possible explanation for this apparent discrepancy might be that catenins are filamentous molecules. Alternatively, the complex might bind a large amount of Triton X-100. Because of the low buoyant density of the detergent, the complex could sediment very slowly. Since in Triton X-114 phase-separation experiments the uvomorulin-catenin complex partitioned into the aqueous phase (Peyri ras et al., 1983) the latter possibility is less likely.

Treatment of the complex with octylglucoside resulted in the specific release of α -catenin. This might be explained by different solubilization properties of octylglucoside since the sugar portion of the detergent is not involved in the action. The fact that even in the absence of α -catenin, β -catenin remains associated with uvomorulin demonstrates a direct interaction between both. In line with this, no solubilization conditions were found in which uvomorulin and α -catenin were detected without β -catenin. Thus, it is presently not known whether α -catenin can interact directly with uvomorulin or only through β -catenin. In addition, the analysis of the assembly of uvomorulin-catenin complex by pulse-

chase experiments revealed that the association of β -catenin takes place immediately after the synthesis of uvomorulin. The association of the newly synthesized α -catenin starts with a lag of about 10 min. One possible explanation for this is that the pool size of α -catenin might be larger than that of the other components. Alternatively, the formation of uvomorulin- β -catenin might be a prerequisite for the incorporation of α -catenin into the complex.

The complex formation with catenins is of crucial importance for the function of uvomorulin. Catenins mediate the connection of uvomorulin with actin and thereby regulate the strength of the uvomorulin-mediated cell adhesiveness as measured in cell aggregation assays (Ozawa et al., 1989, 1990). More recently, it became evident that catenins play an additional role in connecting uvomorulin to other integral membrane proteins such as Na⁺, K⁺-ATPase or cytoplasmic proteins, like fodrin (McNeill et al., 1990). This strongly suggests that catenins are involved in the formation of a submembranous network which links distinct membrane proteins and, upon cell adhesion, redistributes these in a coordinated fashion. Our work described here suggests that catenins might play a similar role when complexed with other cadherins and it will be interesting to see whether the complex formation of catenins with other cadherins is similarly organized.

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