Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 6274–6277, October 1983
Developmental Biology

Uvomorulin: A nonintegral membrane protein of early mouse embryo

(teratocarcinoma/membrane protein/cell adhesion/Ca^{2+})

NADINE PEYRIÉRAS*, FRANÇOIS HYAFIL*, DANIEL LOUVARD*, HIDDE L. PLOEGH†, AND FRANÇOIS JACOB*

*Département de Biologie Moléculaire, Institut Pasteur, 25, rue du Dr. Roux, 75724 Paris Cedex 15, France; and †Institute for Genetics, University of Cologne, Cologne, Federal Republic of Germany

Contributed by François Jacob, July 18, 1983

ABSTRACT **A monoclonal antibody has allowed the characterization of various forms of uvomorulin, a glycoprotein involved in the process of compaction of mouse morula. In addition to various degradation products, uvomorulin exists as a 120-kilodalton exocellular molecule stable at the cell surface. A short-lived 135-kilodalton precursor of uvomorulin has been detected after 10-min pulse labeling. Uvomorulin-like molecules are found on various tissues at various stages of development of the mouse.**

Compaction is a process of adhesion between the blastomeres of the mouse embryo or between embryonal carcinoma (EC) cells. Decompaction is produced by, among other treatments, Fab fragments of rabbit anti-EC IgG (1). The latter effect occurs predominantly through the interaction of IgGs with a surface glycoprotein called uvomorulin (UM), of which an 84-kilodalton (kDa) fragment has been purified after trypsin treatment of EC cell membranes (2). Immunization with this tryptic fragment (UMt) allowed the isolation of a rat monoclonal antibody that can precipitate UMt (3). In this paper, the characterization of UM and its precursor(s) are reported. Arguments are presented to show that UM is deposited as an exocellular protein rather than anchored as an integral membrane protein. Finally, the presence of crossreacting material in the cell membranes from various tissues at various developmental stages of the mouse is demonstrated.

MATERIALS AND METHODS

Cells. Mouse EC line PCC4 Aza R1 and the human melanoma cell line IGR 37 (obtained from Marc Fellous) were used.

Labeling Procedure. For continuous labeling experiments, cells were preincubated 30 min in methionine-free Eagle's normal medium supplemented with 15% fetal calf serum. [^{35}S]Methionine (New England Nuclear) was added at 25 μCi/ml (1 Ci = 37 GBq). Cells were harvested after 12 hr of labeling. For pulse-labeling experiments, a similar procedure was used, but 200 μCi of [^{35}S]methionine per ml was added for 15 or 30 min.

Membrane Preparation. Membrane preparation from PCC4 Aza cells was carried out as described (2) with the addition of 2 mM $CaCl_2$ at 0–4°C in the presence of the following inhibitors: phenylmethylsulfonyl fluoride (0.1 mM), antipaine (1 μg/ml), pepstatine (1 μg/ml), and benzamidine (15 μg/ml) (all from Sigma).

Organs taken from mice (strain 129) were homogenized with a Potter homogenizer in buffer (10 mM Tris, pH 8/2 mM $CaCl_2$ containing the protease inhibitors) and centrifuged at 3,500 rpm in a type JS 5-2 rotor for 15 min. Supernatants were removed and centrifuged at 35,000 rpm in a type 65 rotor for 55 min. Pellets were resuspended in the same buffer, and their protein concentrations were determined by using the Bio-Rad protein assay.

Detergent Extraction. Nonidet P-40-containing buffer was used to prepare lysates from cells or membrane pellets as described (4). Triton X-114-containing buffer was used to prepare lysates from labeled cells. After detergent extraction, the phase separation protocol of Bordier (5) was used. Protease inhibitors and 2 mM $CaCl_2$ were included in all buffers unless otherwise indicated.

Cells Extracted in NaDodSO$_4$. Preparation of cell extracts in NaDodSO$_4$-containing buffer was performed as described (6).

Trypsin Digestion. Trypsin digestion was carried out on detergent lysate prepared from PCC4 Aza membranes at a trypsin (Sigma) concentration of 50 μg/ml in the presence of 2 mM $CaCl_2$ at 37°C for 1 hr. Digestion was stopped by addition of soybean trypsin inhibitor (Sigma) at 100 μg/ml.

Immunoprecipitation. This procedure was carried out as described (7) with a buffer containing 2 mM $CaCl_2$ instead of 5 mM EDTA when necessary. The antibodies used were (*i*) rat monoclonal antibody DE_1, anti-gp84 (3) and (*ii*) mouse monoclonal antibody B 1232 directed against HLA ABC antigens in their complex form.

Charge-Shift Electrophoresis. Charge-shift electrophoresis was performed as described (8) in flat 1% agarose gels. The *Staphylococcus aureus* pellet obtained after immunoprecipitation of radiolabeled UM was eluted in buffer containing 5 mM EDTA. Elution was allowed to take place overnight at 4°C. Gels of these eluates were run at 4°C for 2 hr.

Immunoreplicas. Immunoreplicas were prepared as described (9) by using DE_1 monoclonal antibody at 25 μg/ml, purified rabbit anti-rat IgG at 25 μg/ml, and ^{125}I-labeled protein A (New England Nuclear) at 400,000 cpm/ml.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. In this technique we used the Laemmli buffer system with modifications as described (10). A Tris phosphate stacking gel buffer instead of Tris·HCl was used. Gels were 7.5% acrylamide or linear gradients of 7.5–12% or 5–12% stabilized with sucrose.

RESULTS

Immunoprecipitation of UM from Detergent Lysates of Cells or Membranes. UM was immunoprecipitated by anti-UMt in a detergent lysate of a crude EC cell membrane preparation (Fig. 1). In the presence of Ca^{2+}, two major products having molecular masses of 115–120 and 95–100 kDa (depending on the gel system used) were immunoprecipitated. As will be shown

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Abbreviations: UM, uvomorulin; UMt, tryptic fragment of uvomorulin; kDa, kilodalton; EC, embryonal carcinoma.

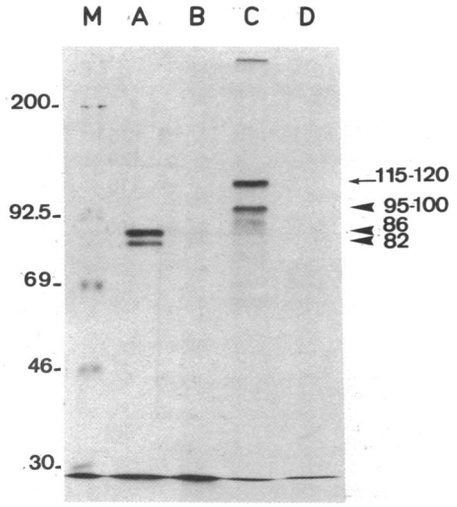

FIG. 1. Immunoprecipitation of UM by DE_1 antibody from a detergent lysate prepared from membranes obtained from radiolabeled PCC4 Aza cells. Detergent extracts of the membranes were subjected to immunoprecipitation, and the immunoprecipitates were analyzed on a 7.5% $NaDodSO_4$/polyacrylamide gel. Lanes: A, detergent lysate treated with trypsin and immunoprecipitated in the presence of 2 mM $CaCl_2$; B, as lane A, but the immunoprecipitation was carried out in the presence of 5 mM EDTA; C, immunoprecipitation was carried out on detergent lysate in the presence of 2 mM $CaCl_2$; D, as lane C but in the presence of 5 mM EDTA; M, size markers (Amersham) in kDa.

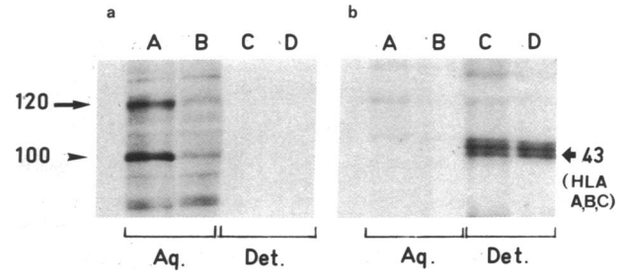

FIG. 2. Phase separation of UM. Continuously labeled IGR 37 and PCC4 Aza cells were mixed and extracted together with a Triton X-114-containing buffer. Immunoprecipitation was carried out on the separated detergent (Det.) and aqueous (Aq.) phases with simultaneous use of DE_1 and B 1232 monoclonal antibodies. Only UM (*a*) and HLA A, B, and C heavy chain (*b*) regions of the autoradiograms are shown. Gels were linear gradients 5–12% acrylamide. Lanes: A, immunoprecipitation was carried out on the aqueous phase in the presence of 2 mM $CaCl_2$; B, as lane A, but in the presence of 5 mM EDTA; C and D, same as lanes A and B, respectively, but on the detergent phase. *a* and *b* are derived from two different gels carrying the same samples. Sizes are shown in kDa.

below, the size of the products obtained did not depend on the method of preparation of the extracts. After trypsin digestion of such a detergent lysate in the presence of Ca^{2+}, two distinct products of 86 and 82 kDa were recovered (Fig. 1). They correspond to the glycoprotein designated gp 84 previously described (2); the gel system used here gave a clear resolution of this doublet.

Ca^{2+} protects UM against complete degradation by trypsin (3) and also might protect it from endogenous proteolysis. When cell lysis in detergent was achieved without Ca^{2+}, the 120-kDa component decreased in amount while the 100-kDa component increased and a few minor bands appeared (data not shown). Because DE_1 antibody recognizes UM only in the presence of 1–2 mM $CaCl_2$ (3), controls were performed in the presence of 5 mM EDTA. Other controls with normal rabbit serum gave similar results (data not shown). It seems, therefore, that the 100-kDa component corresponds to some endogenous degradation product of the 120-kDa molecule. Yet it is not clear whether digestion of the 120-kDa molecule produces the 86-kDa form, while that of the 100-kDa molecule produces the 82-kDa form, or if each of the 120-kDa and 100-kDa molecules gives rise to both the 82- and 86-kDa forms.

Hydrophilic Character of Uvomorulin. During phase separation of a Triton X-114-containing cell lysate, amphipathic membrane proteins are known to partition largely into the detergent phase (5). All fragments of UM immunoprecipitated from a Triton X-114 lysate of continuously labeled cells turned out to partition into the aqueous phase during phase separation (Fig. 2). To check the efficiency of phase separation, PCC4 and IGR 37 cells were mixed and extracted simultaneously with Triton X-114-containing buffer. After phase separation, HLA A and B antigens, integral membrane proteins (11), were enriched in the detergent phase, while UM was found almost exclusively in the aqueous phase (Fig. 2). These results suggest that the 120-kDa molecule is hydrophilic. Because the 120-kDa form might be an artefactual degradation product of native UM, rapid lysis of PCC4 Aza cells was performed in a $NaDodSO_4$-containing buffer in the presence of an excess of protease inhibitors, and this preparation was analyzed by immunoreplica. The largest product recognized by anti-UMt in the presence of calcium was the 120-kDa product previously identified in a Triton X-114-containing cell lysate (Fig. 3).

To further document the hydrophilic character of UM, charge-shift electrophoresis was performed on material eluted in EDTA-containing buffers from immunoprecipitated UM. The two forms of UM, 120 kDa and 100 kDa in roughly equal amounts, were found to behave similarly in the different detergent-containing buffers (data not shown). This result confirms the hydrophilic character of UM.

In order to detect events occurring during UM biosynthesis,

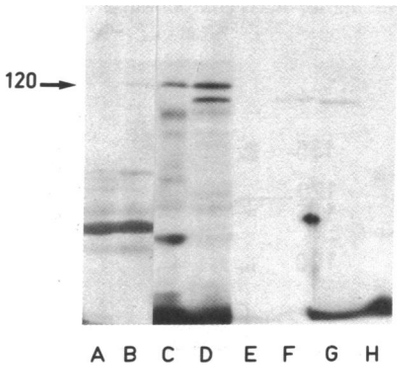

FIG. 3. Immunoreplica analysis of PCC4 Aza cell extracts. Three different methods of preparation of PCC4 Aza cell extracts are compared by immunoreplica; size is shown in kDa. Lanes: A, products immunoprecipitated by anti-UMt in the presence of 5 mM EDTA have been resolved by 7.5% $NaDodSO_4$/polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter, which was stained with red Ponceau S; B, as lane A but in the presence of 2 mM $CaCl_2$; C, an aliquot of a PCC4 Aza membrane preparation containing about 80 μg of protein was analyzed with all buffers containing 2 mM $CaCl_2$; D, PCC4 Aza cells were rapidly lysed in a $NaDodSO_4$-containing buffer (an aliquot of this lysate was analyzed as in lane C); E and F, same as lanes C and D, respectively, but with normal rabbit serum instead of anti-UMt monoclonal antibody; G and H, same as lanes D and C, respectively, but with all buffers containing 5 mM EDTA. All samples were analyzed on a single gel and transferred to a single nitrocellulose sheet, which was then cut in four pieces to produce the four experimental A–D and control E–H lanes. The lower major band (≈110 kDa) appears to be nonspecific because it is present in controls.

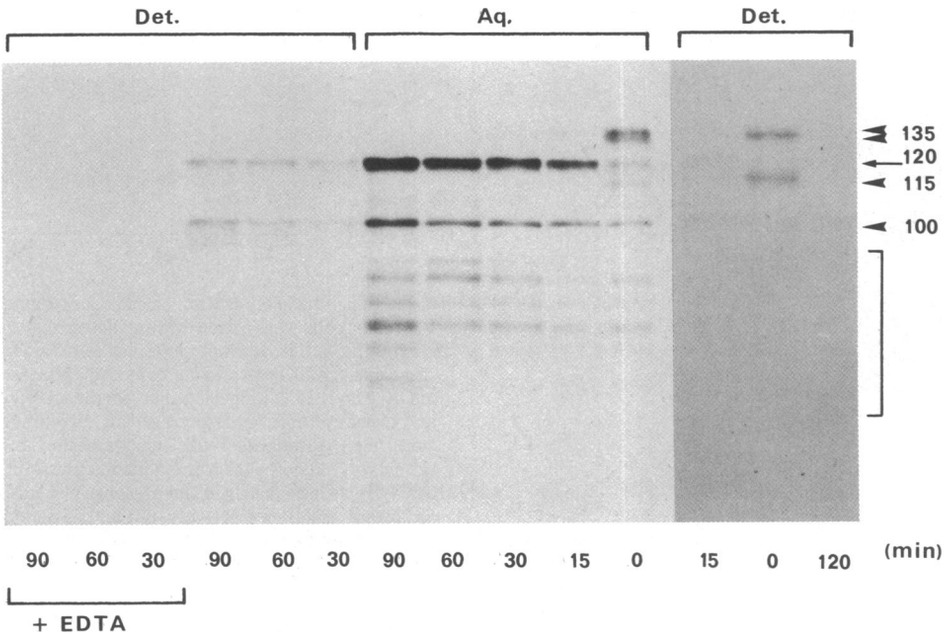

FIG. 4. Pulse–chase analysis of UM synthesis in PCC4 Aza cells. Cells were pulse–labeled for 10 min and chased for 0, 15, 30, 60, 90, or 120 min as indicated. Cells were extracted with a Triton X-114-containing buffer. Immunoprecipitation with anti-UMt was performed on the aqueous (Aq.) and the detergent (Det.) phases in the presence of 2 mM $CaCl_2$ or 5 mM EDTA (+ EDTA) where indicated. Gels were linear gradients of 7.5–12% acrylamide. The minor bands indicated by a bracket are possible degradation products of UM. The products immunoprecipitated from the detergent phase are shown overexposed in order to visualize the residual amount of 100-kDa and 120-kDa molecules. All controls performed in the presence of 5 mM EDTA gave results similar to those shown. Sizes are shown in kDa.

pulse–chase experiments were performed. After a 10-min (Fig. 4) or 30-min (data not shown) pulse-labeling, immunoprecipitation at time 0 gave rise, in addition to the 120- and 100-kDa forms, to a 135-kDa doublet and to a 115-kDa molecule. After a 15-min chase (10-min pulse), the 115-kDa form and the component of the doublet with the lower molecular mass had disappeared. The higher-mass component of the 135-kDa doublet was still present in a small amount after the 15-min chase but had disappeared completely after the 60-min chase. While the amount of the upper band of the 135-kDa doublet decreased with time, the amount of the 120-kDa band increased and other minor bands appeared. Both the 115-kDa band and the lower band of the 135-kDa doublet were quantitatively recovered in the detergent phase after phase separation in Triton X-114 (Fig. 5). These amphipathic components observed after a 15-min chase of a 10-min pulse are unlikely to be already present at the surface (12). They might rather reside in the rough endoplasmic reticulum or early Golgi apparatus. The fact that some transient forms of UM partition into the detergent phase gives additional support to the hydrophilic character of the 120-kDa molecule.

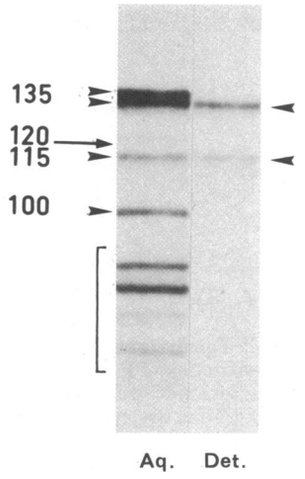

FIG. 5. The 135-kDa precursor of UM consists of two molecular forms. PCC4 Aza cells were labeled for 10 min in the presence of 1 mM deoxymannojirimycin, a substance which inhibits conversion of high-mannose to complex type N-linked glycans (unpublished results) and extracted in a Triton X-114-containing buffer. Immunoprecipitation with anti-UMt in the aqueous (Aq.) and detergent (Det.) phases shows a clear resolution of the 135-kDa doublet, as indicated by the two arrowheads. The 115-kDa and 100-kDa molecules likewise are indicated by arrowheads. The 120-kDa molecule, barely visible in the aqueous phase, is indicated by an arrow. As in Fig. 4, the bracket indicates degradation products of UM.

Presence of Crossreacting Molecules at the Surface of Various Cell Types at Various Times of Development. Membranes were prepared from lung, liver, intestine, and brain obtained from 17-day mouse embryos and from newborn and adult mice. They were analyzed by the immunoreplica technique. DE_1 monoclonal antibody detected multiple products in all cases except brain. In each organ, some differences were apparent between the three stages, possibly due to degradation (Fig. 6). Further analysis is required to provide insight into the relationship between these various molecules and UM as well as between these molecules themselves.

DISCUSSION

UM appears to exist at the surface of EC cells as a 120-kDa molecule that can be degraded into a variety of fragments. The 120-kDa molecule does not seem to contain a hydrophobic domain that would give the molecule a membrane anchor because the two techniques used show that it lacks micelle-binding properties. Furthermore, significant quantities of the 120-kDa and 100-kDa forms are released by incubation of cells with 5 mM EDTA (unpublished data), supporting the argument that UM is not an amphipathic structure.

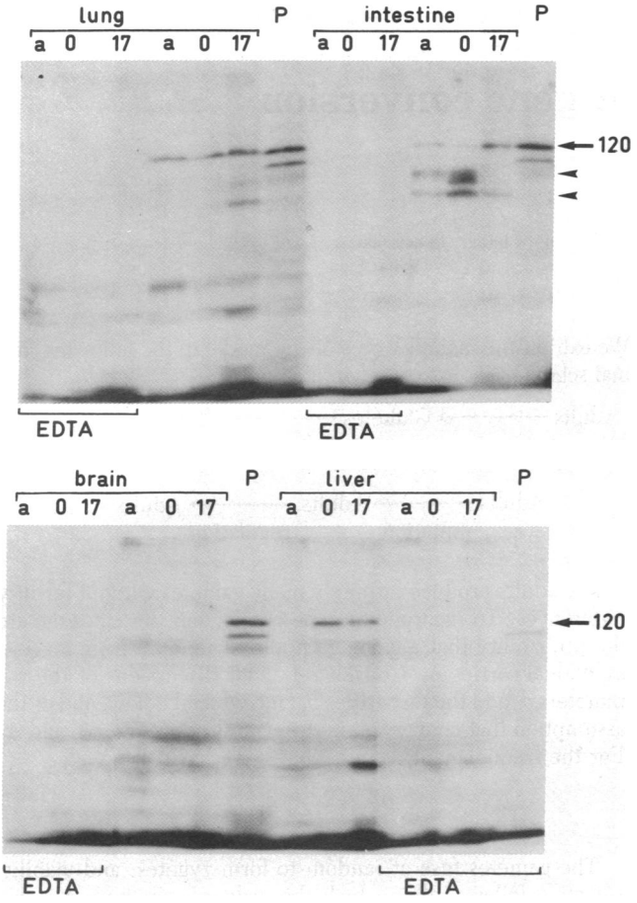

FIG. 6. Expression of UM-like molecules on various mouse organs at different stages of development. Aliquots of crude membrane preparations obtained from different mouse organs were analyzed by immunoreplica. Brain, intestine, liver, and lung membrane preparations, as indicated, were compared with the PCC4 Aza cell preparation (P) described in Fig. 3, lane D. Three stages of development are displayed side by side: 17-day embryos (lanes 17), newborn mice (lanes 0), and adult mice (lanes a). Controls consisted of incubations with antibody in the presence of 5 mM EDTA as indicated (EDTA). The 120-kDa molecule is indicated by an arrow; the major products recognized by anti-UMt in the presence of calcium are indicated by arrowheads.

In pulse–chase experiments, a short-lived 135-kDa doublet and a 115-kDa form are observed. They exhibit the Ca^{2+}-dependent conformational change characteristic of UM. Because the lower 135-kDa and the 115-kDa components have a much shorter lifetime than the higher 135-kDa component, the latter disappearing progressively as the stable 120-kDa form increases in amount, it is possible to construct a hypothetical biosynthetic pathway of UM. Only the lower band of the 135-kDa doublet and the 115-kDa molecule partition into the detergent phase of a Triton X-114 lysate and, therefore, could be different forms of a UM precursor containing hydrophobic components. Then, the upper band of the 135-kDa doublet could correspond to the UM precursor after elimination of these hydrophobic features. There is no precedent for retention of the typically hydrophobic NH_2-terminal signal peptide in pulse–chase experiments of this type. It seems more likely that a proteolytic cleavage other than removal of the NH_2-terminal peptide, if present, is taking place. In addition, other reactions, such as sugar modifications, might take place and could explain the slight difference in molecular mass between the two bands of the 135-kDa doublet. UM is known to contain at least one N-linked glycan, probably of the complex type (13). The 135-kDa doublet persists when pulse–chase experiments are performed in the presence of deoxymannojirimycin (1,5-dideoxy-1,5-diimino-D-mannitol), a compound reported to inhibit conversion of high-mannose- to complex-type oligosaccharides (unpublished results). Thus, the difference in size between the two components of the 135-kDa doublet cannot be the consequence of such a conversion.

The distribution of UM is not confined to the early embryo. Molecules recognized by anti-UMt have been found on membranes prepared from liver, intestine, and lung (but not brain) taken from mice at various stages of development. Evidence for the presence of Ca^{2+}-dependent cell adhesion molecules have already been presented not only for teratocarcinoma cells (2, 14) but also for embryonic chicken liver (15). It seems likely that there exists a whole family of such molecules whose relationships remain to be defined.

We thank Marc Fellous for supplying monoclonal antibody B1232 and Didier Hatat for preparing labeled IGR37 cells. This work was supported by grants from the Centre National de la Recherche Scientifique (LA 269), the Fondation pour la Recherche Médicale, the Institut National de la Santé et de la Recherche Médicale (CRL 801012), the Ligue Nationale Française contre le Cancer, and the Fondation André Meyer. H.L.P. is supported by the Deutsche Forschungsgemeinschaft through SFB 74.

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