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Immunofluorescent and Immunochemical Evidence for the Expression of Cytovillin in the Microvilli of a Wide Range of Cultured Human Cells

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We have previously purified a M_r 75,000 protein, cytovillin, from cultured human choriocarcinoma cells (JEG-3) and shown that this protein was specifically confined to the microvillus membrane of these cells. I have now studied the expression and the subcellular distribution of cytovillin in eighteen normal and transformed human cell lines and strains by using immunoblotting and indirect immunofluorescence microscopy. In all cell types, cytovillin was highly enriched in cell surface protrusions. When cell types were ranked according to their staining intensity, choriocarcinoma was highest, then amniotic epithelial cells, other choriocarcinoma cells and tumor cells, and finally fibroblastoid cells. The latter only gave faint diffuse fluorescence on the plasma membrane and, occasionally, on the microvilli. However, detergent extracts of all cell types could be shown to contain cytovillin by the use of immunoblotting techniques. Metabolic pulse-chase labelling experiments with JEG-3 cells demonstrated synthesis of cytovillin as a single-chain polypeptide. No precursor forms or specific proteolytic cleavage products could be seen either by immunoblotting or immunoprecipitation. The protein was found to be very stable with a biologic half-life of about 25 hours. The pI determined by isoelectric focusing was 6.1. These results were consistent with cytovillin being an integral component of the microvilli and other surface extensions of all human cell types examined.

Key words: bleb, membrane protein, microvillus

We have previously purified to homogeneity a M_r 75,000 protein, cytovillin, from cultured human JEG-3 choriocarcinoma cells [1]. This protein was originally detected by using rabbit polyclonal antibodies raised against a synthetic peptide whose sequence was derived from a cloned human endogenous retroviral nucleotide sequence. The peptide-specific antibodies detected cytovillin in syncytiotrophoblasts of first-trimester human placentas, in choriocarcinoma tissues, in cultured choriocarcinoma cells [2], and in renal cell adenocarcinoma tissues [3]. Triton X-100 extracted almost all cytovillin from the JEG-3 cells [4], and the cytovillin was enriched in the hydrophilic phase [1] of the Triton X-114 two-phase separation of Bordier [5].

By using rabbit polyclonal antibodies raised against purifed cytovillin, the protein has been recently localized in the JEG-3 cells at light and electron microscopic level.

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By using immunoperoxidase and immunoferritin electron microscopy, cytovillin was found to be specifically confined to microvillus membrane. Nearly all (87%) of the plasma membrane-bound ferritin label was calculated to be in the microvilli. Immunofluorescence and the immunoferritin electron microscopy also showed that the antigenic site(s) of cytovillin were on the cytoplasmic side of the plasma membrane [4].

So far only a few microvillus-specific proteins have been described, and their distribution in different cell types in vitro has not been well characterized. Most of the microvillus proteins known are hydrolytic enzymes or transport proteins, which have been purified from the brushborders of epithelial cells of intestine and kidney. These include, for example, sucrase-isomaltase, maltase-glucoamylase, lactase-phlorizin hydrolase, and several peptidases [6–10].

The major structural proteins of microvillin are actin (43 kd) [11], villin (95 kd) [12,13], fimbrin (68 kd) [14] and calmodulin (17 kd) [15]. The core of the microvillus is composed of a bundle of uniformly polarized actin filaments, whose barbed ends are embedded in a dense plaque at the tip of the microvillus. Villin and fimbrin cross-link the actin filaments into bundles [16]. Calmodulin-binding protein (110 kd) [17–19] and 140-kd integral membrane protein [20] probably connect the actin filaments of the core to the membrane of the microvilli.

In this report, the expression and the subcellular distribution of cytovillin were studied by immunoblotting of cell extracts and indirect immunofluorescence microscopy. Cytovillin was found to be expressed in a wide variety of normal and transformed human cell lines and strains and, within each cell, to be highly restricted to cell surface extensions, including microvilli, blebs, and long cell surface protrusions. According to metabolic pulse-chase radioisotope experiments, cytovillin was synthesized as a single polypeptide in the JEG-3 cells, and its biologic half-life was about 25 hours.

MATERIALS AND METHODS

Cell Cultures

All cell lines and strains (see Table I) were of human origin. The following cell lines were obtained from the American Type Culture Collection: A-204, HTB 82; A-549, CCL 185; BeWo, CCL 98; CaSki, CRL 1550; G-361, CRL 1424; HOS, CRL 1543; HT-1080, CCL 121; JEG-3, HTD 36; RD, CCL 136; SK-N-SH, HTB 11; and Wi-38, CCL 75. For TuWi and A-9733 cells, see ref. 21. The HAL, HEL, HME, and HES were prepared by conventional tissue trypsinization methods and propagated as described previously [22]. HuA cells were prepared from amniotic membranes of full-term post partum placenta as described previously [23,24]. The cells were grown on plastic dishes with or without glass coverslips in Eagle's minimal essential medium (MEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, and 5 μg/ml streptomycin.

Anti-M_r 75 000 Serum

Antiserum against purified cytovillin [1] was raised in a rabbit by using four subcutaneus immunizations, 25 μg cytovillin per injection in Freund imcomplete adjuvant at 2-week intervals. The specifity of the antiserum has been described elsewhere [4]. Before immunization, serum was collected from the same rabbit.

For immunofluorescence microscopy, the antibodies against cytovillin were affinity purified by the following procedure. Cytovillin was immunoprecipitated from extracts

TABLE I. Occurrence of Cytovillin in Different Cell Lines and Strains

Cell strain	Intensity of immunofluorescence[a]	Immunoblotting
JEG-3 (Choriocarcinoma)	+ + +	+
BeWo (Choriocarcinoma)	+ + +	+
HuA (Amniotic epithelial cells)	+ + +	+
CasCi (Uterine cervical carcinoma)	+ +	+
A-549 (Lung carcinoma)	+ +	+
HT-1080 (Fibrosarcoma)	+	+
TuWi (Wilms' tumor)	+	+
HOS (Osteosarcoma)	+	+
A-204 (Rhabdomyosarcoma)	+	+
G-361 (Malignant melanoma)	+	+
SK-N-SH (Neuroblastoma)	+	+
RD (Rhabdomyosarcoma)	+	+
A-9733 (Fibrosarcoma)	±[b]	+
HAL (Adult lung fibroblast)	±[b]	+
HME (Adult skin fibroblast)	±[b]	+
HEL (Embryonal lung fibroblast)	±[b]	+
HES (Embryonal skin fibroblast)	±[b]	+
Wi-38 (Embryonal lung fibroblast)	±[b]	+

[a]The intensity of the fluorescence label: + + + very bright, + + bright, + weak, − background.
[b]Only occasional microvilli and blebs were detected.

of the cell layers of 10 64 cm^2 JEG-3 cultures with the polyclonal anti-cytovillin serum (see Immunoprecipitation). The precipitated proteins were separated by sodium dodecylsulfate–polyacrylamide (10%) gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to nitrocellulose filters (see Immunoblotting). After transfer, the nitrocellulose sheets were treated with Ponceau red, and the nitrocellulose fragments corresponding to cytovillin were excised. About 15 μg of pure cytovillin was recovered by comparing the staining intensity to that of a known amount of bovine serum albumin used as standard. The nitrocellulose fragments were collected in a microfuge tube and washed several times with TEN-Tween buffer (50 mM Tris/HCl, pH 7.0; 5 mM EDTA; 150 mM NaCl; 0.05% Tween 20). The cytovillin serum was then applied at a dilution of 1:3 in TEN-Tween buffer and incubated for 3 hr at 37°C in an end-over mixer. After extensive washing with the TEN-Tween buffer, the bound antibodies were eluted in 500 μl of 0.1 M glycine, pH 2.5. The eluate was immediately neutralized to pH 7.5 with solid Tris, and a 10-μg amount of carrier bovine serum albumin (BSA) was added. The mixture was dialyzed overnight against 10 mM Tris/HCl, pH 7.5, and freeze-dried. The final yield of the antibodies was about 5 μg per 15 μg of antigen. The freeze-dried antibodies were dissolved in distilled water and the final concentration of the antibodies was adjusted to 40 μg/ml.

Polyacrylamide-Gel Electrophoresis and Immunoblotting

Cell cultures were washed twice with phosphate-buffered saline (PBS) and solubilized in Laemmli sample buffer, and the reduced samples were applied to a 10% homogenous SDS–PAGE gel [25]. After separation, the proteins were transferred electrophoretically from gels to nitrocellulose sheets (0.45-μm pore size, BA-85; Schleicher & Schüll Co., Dassel, FRG) as described previously [26], and the sheets were incubated with the rabbit anti-cytovillin serum or control serum (1:2000 in TEN-Tween buffer)

for 1 hr at 37°C. Immunoreactive protein bands were detected with peroxidase-conjugated anti-rabbit IgG (DAKO, Copenhagen, Denmark; 1:200 dilution in TEN-Tween buffer). For fluorography, the gels were treated first for 30 min in 10% (v/v) acetic acid and then for 30 min in "Amplify" (Amersham International, England). Dried gels were then exposed to Kodak X-Omat film.

Metabolic Radioisotope Labelling

Subconfluent JEG-3 cell cultures grown on plastic dishes (64 cm^2) were first kept for 1 hr in methionine-free Minimal Essential Medium (MEM) without serum. Thereafter, 20 uCi/ml of L-^{35}S-methionine (1,170 Ci/mmol; Amersham) was added, and the cells were kept in culture for 15 min. After the radioactive pulse, the culture medium was replaced by MEM containing a 50-fold excess of the normal amount of unlabelled L-methionine and 10% FCS. The cultures were then incubated for a period ranging from 15 min to 55 hours.

Immunoprecipitation

Metabolically labelled JEG-3 cells grown on 64 cm^2 plastic dishes were washed once with PBS and solubilized in a buffer containing 0.1% (w/v) SDS, 0.5% (v/v) Triton X-114, 50 mM Tris/HCl, pH 8.0; 150 mM NaCl; and 1 mM phenylmethanesulphonyl fluoride (500 μl). Insoluble debris was removed by centrifugation at 9,000 *g* for 2 min. Ten microliters of the rabbit control serum were then added to the supernatant; and after 10 min, 70 μl of a 10% (w/v) suspension of washed protein-A Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) in 50 mM Tris/HCl, pH 8.0, 150 mM NaCl. After a further 10 min, the beads were collected by centrifugation at 9,000 *g* for 1 min, and a 5-μl amount of the immune serum was added to the supernatant. After an overnight incubation at +4°C, protein-A Sepharose beads were added as above, and the mixture was incubated for 60 min at +4°C in an end-over mixer. The precipitates were washed 4 times with 50 mM Tris/HCl, pH 8.0; 150 mM NaCl, 0.5% (v/v); Triton X-114; and 0.1% (w/v) SDS and once with 50 mM Tris/HCl, pH 8.0; and 150 mM NaCl before analysis on 10% polyacryamide gels.

Isoelectric Focusing

Isoelectric focusing was performed using a PhastSystem apparatus (Pharmacia) and polyacrylamide gels (pI 3–9) according to the manufacturers instructions. The proteins were visualized by automated silver staining of the gels [27] in the PhastSystem's development unit.

Immunofluorescence Microscopy

The cell cultures on glass coverslips were fixed with freshly dissolved 3.5% (w/v) paraformaldehyde (15 min, 20°C). After washing with Dulbecco's PBS, containing 2 mg/ml bovine serum albumin, and 0.05% Saponin (PBS/BSA/Saponin), the cells were treated with affinity-purified anti-cytovillin antibodies (4 μg/ml) or with the control serum at a dilution of 1:100 in PBS/BSA/Saponin. The cell-bound antibodies were detected with purified sheep IgG raised against rabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Wellcome, Beckenham, England) (1:40 as above). The fluorescence was observed with a Leitz Dialux 20 microscope fitted with a 63× oil immersion objective. Photomicrographs were taken on Agfapan 400 film.

RESULTS

Immunoblotting

The presence of cytovillin in different types of cultured human cells was demonstrated by immunoblotting of cell extracts and indirect immunofluorescence microscopy. No differences could be seen in immunoblotting with the polyclonal antibodies between the relative molecular weight of cytovillin in the different cell lines and strains. No protein bands were detected when the control serum was used instead of the anti-cytovillin serum (Fig. 1). The intensity of the specific peroxidase reactions was highest with extractions of choriocarcinoma (JEG-3, BeWo) and amniotic epithelial (HuA) cells. A relatively weak signal was detected in the immunoblotting of the other cell types.

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy with affinity-purifed antibodies against the immunoprecipitated cytovillin were used to study the subcellular localization of cytovillin in the different cell types. After fixation, the cells were permeabilized with Saponin, which was shown to preserve the ultrastructure of the cells better than methanol treatment. Both permeabilization methods gave a similar pattern of fluorescence [4], and the antibodies gave an excellent signal-to-background ratio. The specific microvillus (see below) fluorescence could also be seen, if the cells were treated with 0.1% (v/v) Triton X-100 after fixation (data not shown).

In most cells, the fluorescent label appeared in cell surface extensions, such as microvilli and blebs. However, the intensity of the label varied among different cell types (Table I). The fluorescent label was most prominent in epithelial cells: BeWo (choriocarcinoma), A-549 (lung carcinoma), CasCi (uterine cervical carcinoma), and HuA (amniotic epithelial cells). In BeWo, A-549, and HuA cells, cytovillin was observed

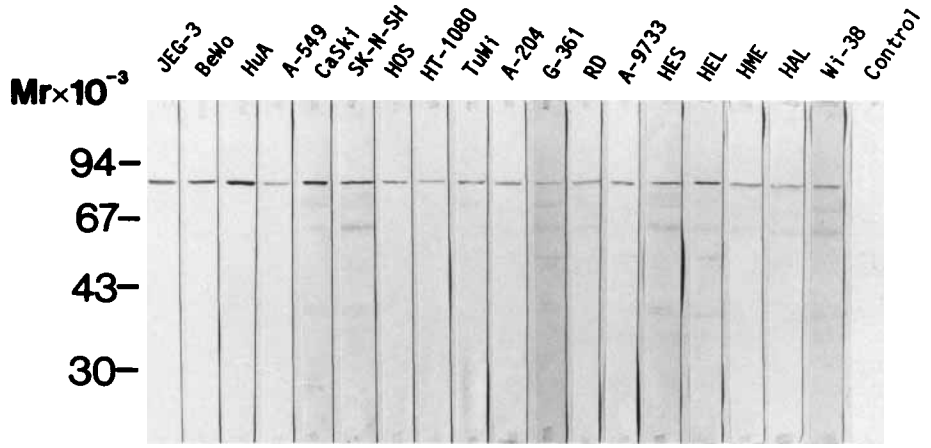

Fig. 1. Immunoblotting analysis of extracts from the cell layers of different human cell lines and strains. The cells were lysed with Laemmli's sample buffer and the proteins were run under reducing conditions on a 10% homogenous gel. From the gel, the proteins were transferred electrophoretically to nitrocellulose sheets, which were then treated with rabbit antiserum to cytovillin. The control strip on the right carries an extract of the JEG-3 cells treated with the control serum. Molecular weight markers are on the left.

in relatively short microvilli on the apical cell surfaces (Fig. 2) similar to those seen in JEG-3 cells. There were no significant differences among each cell type in culture in the amount of the microvilli. No other cytovillin-positive intra- or extracellular structures were visible. When anti-cytovillin antibodies were substituted with the control serum, only negligible background fluorescence was see (Fig. 2). Also in TuWi (Wilms' tumor), HT-1080 (fibrosarcoma), A-204 (rhabdomyosarcoma), RD (rhabdomyosarcoma), and G-361 (malignant melanoma) (Fig. 2), the antigen was mainly detected in similar short microvilli, although the intensity of the fluorescence label was considerably weaker in these cells than in the epithelial cells. Occasionally cytovillin was detected in blebs on cell surfaces (Fig. 3). In CasCi (uterine cervical carcinoma), SK-N-SH (neuroblastoma), and HOS (osteosarcoma), cytovillin was also observed in long spiky cell surface protrusions (Fig. 3).

Four different fibroblastic cell lines and strains were analyzed: HAL (adult lung fibroblasts), HME (adult skin fibroblasts), HEL (embryonal lung fibroblasts), HES (embryonal skin fibroblasts), and Wi-38 (embryonal long fibroblasts). In these cells, only faint diffuse plasma membrane fluorescence and scattered microvilli, as well as blebs, were detected on cell surfaces (Fig. 3). A-9733 fibrosarcoma cells, which had a fibroblastoid morphology, gave a similar staining pattern. The fibroblasts featuring microvilli were usually more rounded than those without microvilli.

The results showed that cytovillin is expressed in a wide variety of normal and transformed cell lines and strains, where it is strictly localized to cell surface extensions.

Metabolic Labelling with Radioisotope

To study the biosynthesis and stability of cytovillin in vitro, JEG-3 cells were labelled metabolically for 15 min with L-^{35}S-methionine, followed by a period of 15 min to 55 hr with a 50-fold excess of unlabeled methionine in the presence of 10% (v/v) FCS in the culture media. The labelled cytovillin was immunoprecipitated with the anti-cytovillin serum and analyzed on SDS-PAGE under reducing conditions followed by fluorography. Only one M_r 75 000 protein band was observed in the labelled immunoprecipitates (Fig. 4), indicating that cytovillin was synthesized as a single polypeptide in the JEG-3 cells. No specific proteolytic cleavage products were evident. These results agreed with the immunoblotting, in which only one polypeptide band was detected. From the slow rate of disappearance of radioactive label in the immunoprecipitates obtained after different chase periods, it was apparent that cytovillin was very stable in vitro. The biological half-time of cytovillin was estimated to be about 25 hours.

Isoelectric Focusing

The isoelectric point of the purifed protein was determined by using a PhastSystem apparatus with pI 3-9 polyacrylamide gels. After staining the gels with a sensitive silver staining method, only one band, with pI 6.1, was found (Fig. 5). The result suggests that the purified cytovillin had no charge heterogeneity.

DISCUSSION

To study the distribution of cytovillin in different cell types in vitro, 18 normal and transformed human cell lines and strains were analyzed by immunoblotting of the cell extracts and immunofluorescence microscopy. Quite unexpectedly, cytovillin was found to be expressed in every cell line and strain. In all cell types, the protein was highly localized in cell surface extensions, including microvilli, blebs, and long spiky

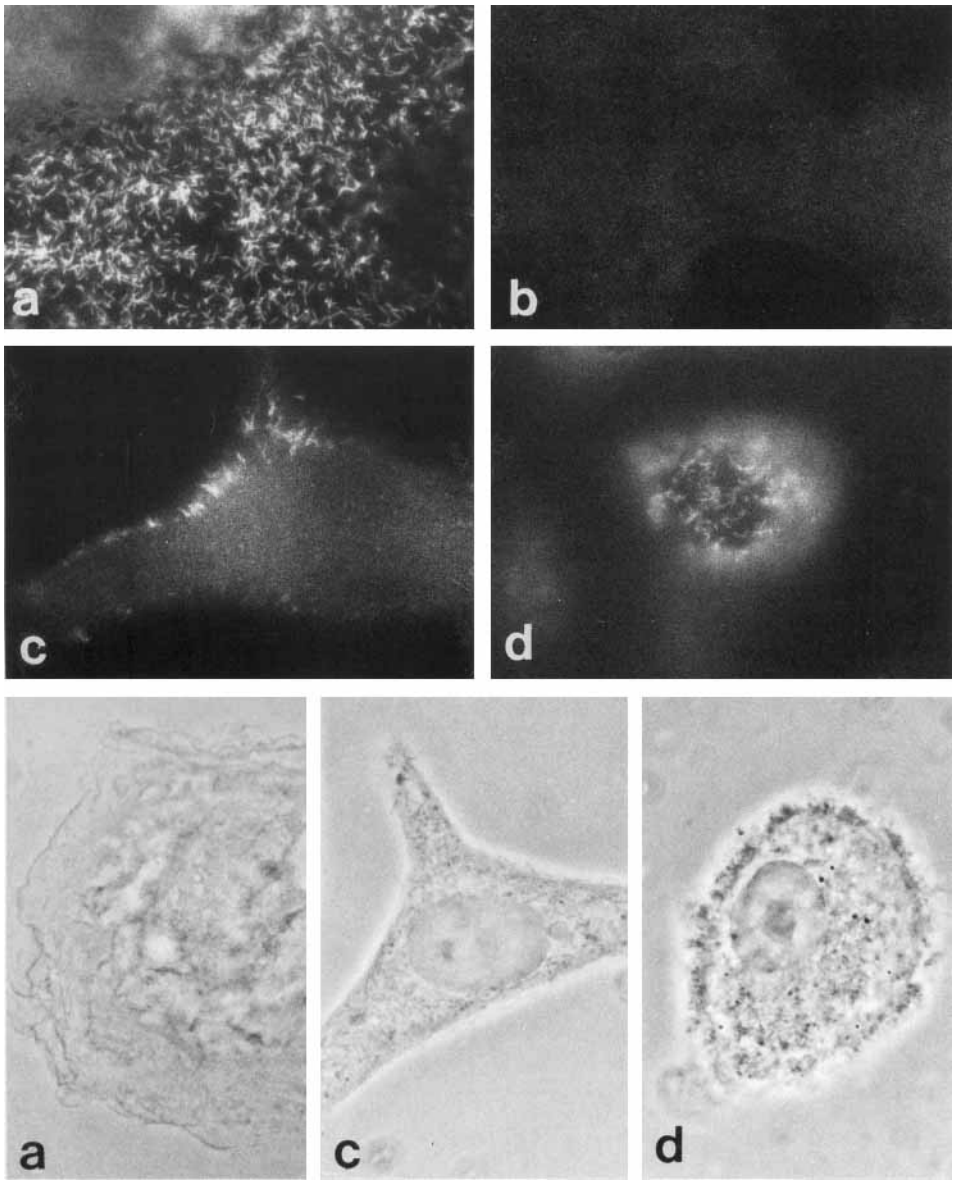

Fig. 2. Immunofluorescence staining of cells by using affinity purified rabbit antibodies against cytovillin (**a,c,d**) or the control rabbit serum (**b**). Figures (**a**) and (**b**) represent BeWo, (**c**) G-361 and (**d**) A-549 cell cultures. The fluorescent label can be seen in relatively short microvilli on cell surfaces (upper figures). The lower figures show the corresponding cells in phase contrast microscopy. Magnification (a–d), ×1,000.

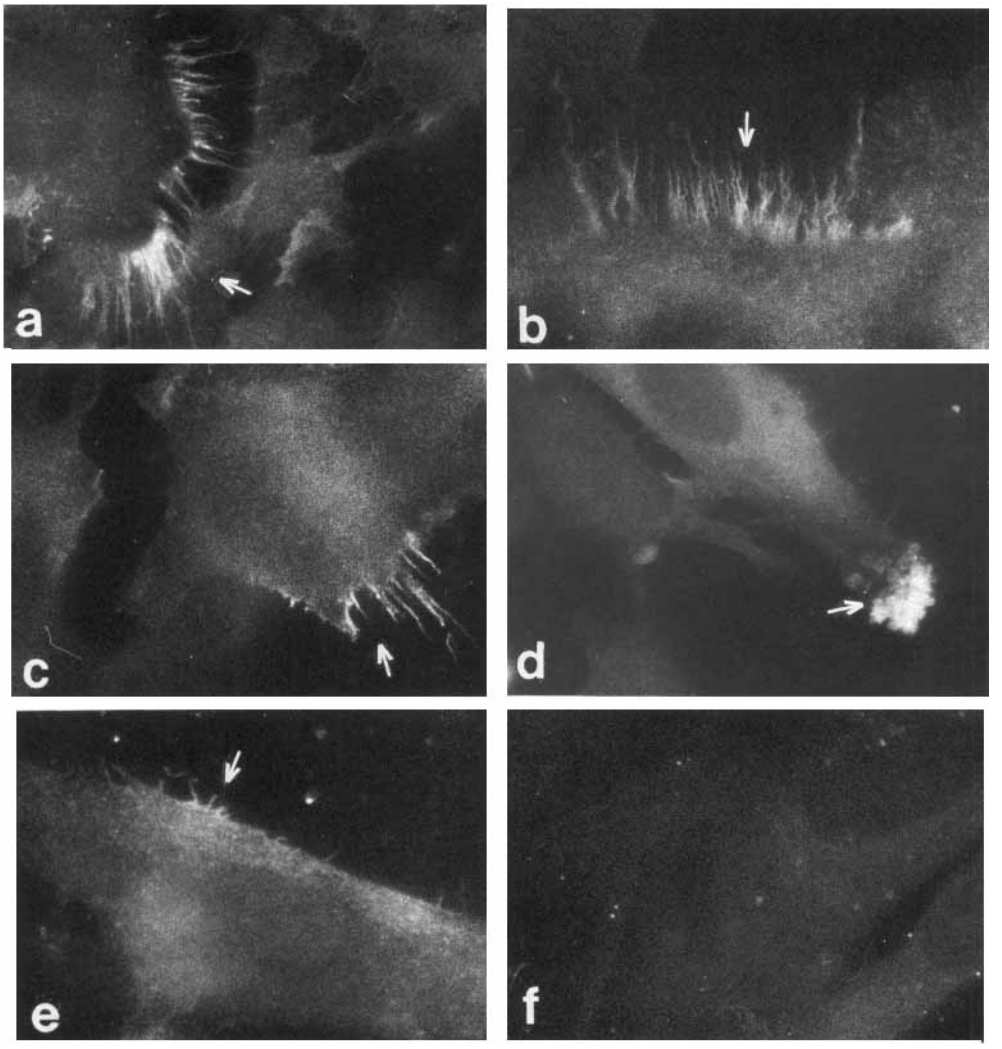

Fig. 3. Immunofluorescence staining of HOS (**a**), CasCi (**b**), SK-N-SH (**c,d**), and HES (**e,f**) cell cultures by using rabbit anti-cytovillin antibodies (**a–e**) or the control serum (**f**). Cytovillin can be seen in long spiky cell surface protrusion (**arrows, a–c**) or in blebs (**arrow, d**). In HES, only faint diffuse plasma membrane fluorescence and occasional microvilli (**e, arrow**) are apparent. Magnification (a–f), ×1,000.

cell surface protrusions. The fluorescent label was most prominent in choriocarcinoma cells and amniotic epithelial cells, and the intensity of the label correlated with the intensity of bands detected after immunoblotting of cell extracts. In fibroblastoid cells, only faint plasma membrane fluorescence, limited often to scattered microvilli and blebs, were seen on cell surfaces. The few microvilli-containing fibroblasts were usually more rounded than those without microvilli, and these could have been in the mitotic stage of the cell cycle.

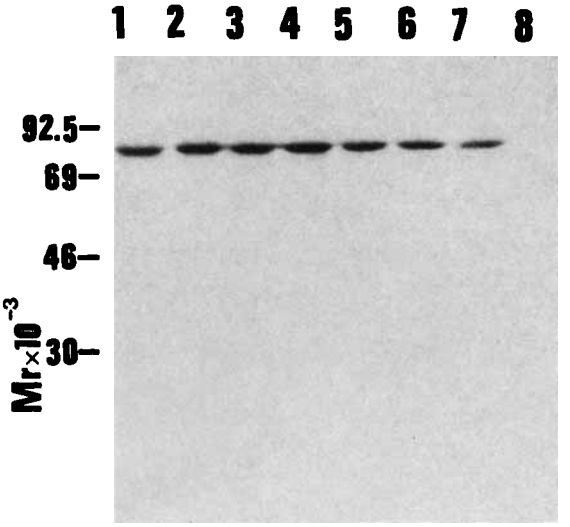

Fig. 4. Pulse-chase labelling of cytovillin. JEG-3 cells were labelled metabolically for 15 min with L-^{35}S-methionine and chased with excess unlabeled methionine for 0–55 hr. The labelled cytovillin was immunoprecipitated with rabbit antiserum to cytovillin and the immunoprecipitates were analyzed on a 10% SDS-PAGE gel followed by fluorography. **Lanes 1–8** represent 0 min, 15 min, 30 min, 1 hr, 2 hr, 7 hr, 24 hr, and 55 hr chase periods, respectively. Molecular weight markers are on the left.

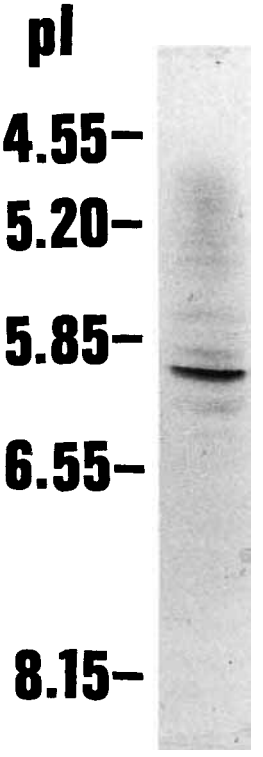

Fig. 5. Isoelectric focusing of purified cytovillin on a pI 3–9 polyacrylamide gel. The proteins were visualized by silver staining of the gel. The positions of the marker proteins (Pharmacia) are indicated on the left.

The biochemical function of cytovillin is unknown. While the known structural proteins of the microvilli are mostly Triton X-100 insoluble [11–20], on the contrary, cytovillin could be almost completely released from the cells with nonionic detergent under conditions which have been reported to leave the cytoskeleton intact [4]. In the immunoferritin and the immunoperoxidase microscopy we showed that the protein was localized to the plasma membrane, not to the core of the microvilli, and all microvilli appeared to have cytovillin [4]. However, cytovillin is exposed only on the cytoplasmic side of the plasma membrane, since the protein could not be radioiodinated by the lactoperoxidase method (data not shown), and the antigenic epitopes were observed to be under the plasma membrane [4]. When the Triton X-114 two-phase separation was applied, as described by Bordier [5], cytovillin was found to be concentrated in the hydrophilic phase, suggesting that the protein is not an integral membrane protein [1]. In summary, thse results were consistent with cytovillin being a peripheral membrane protein, associated with the cytoplasmic surface of the microvillus plasma membrane.

The formation of microvilli probably starts at actin-containing microdomains of the plasma membrane that later become the tips of the microvilli [7]. The 140,000-dalton glycoprotein [20] and the 110,000-dalton protein [17–19] connect the actin filaments of the core to the membrane of microvilli. It is possible that cytovillin may also be involved in the formation of microvilli and could be relocated from the smooth plasma membrane into the membrane of the growing microvilli.

So far, it has not been determined whether cytovillin is associated with any other known protein. In gel filtration of the Triton X-100-solubilized cellular proteins, cytovillin behaved as a single polypeptide [1]. However, this does not exclude the possibility that cytovillin had specific but weak interactions with some other microvillus-specific proteins in cells. For example, it has been reported that the microvillus membrane contains an intermediate form of actin [28], but its relationship to the filamentous actin or its interaction with cytovillin is unknown.

Quite little is known about the distribution of the known microvillus proteins in different cell types in vitro. Fimbrin was detected in microvilli, microfilamentous meshwork, and membrane ruffles in rat mammary cells and chicken embryo fibroblast [14]. Two reports described a minor 80,000 dalton component of microvillus core, which may be identical. This p80 has been detected in blebs, microvilli, and retraction fibers in chicken embryo fibroblasts and mouse 3T3 cells [29]. A p81 protein tyrosine kinase substrate is homologous to p80 protein, and it has been shown to be concentrated in microvilli and retraction fibers of A431 cells [30]. Villin could not be detected in cultured cells, but when it was microinjected into the cells, the protein was found to be associated with the microfilamentous structures [31]. The immunofluorescence and immunoblotting results obtained above demonstrated that the expression of cytovillin is not restricted to trophoblastic cells. The result also indicate that the microvillar location of the protein is not dependent on the cell type, but is probably an inherent property of the cytovillin molecule.

So far, cytovillin has been found to be expressed in vivo in placenta, choriocarcinomas [2] and renal cell adenocarcinoma tissues [3], and the author and co-workers are currently making a large-scale study of its expression in other human normal and cancer tissues. The previous negative results in immunohistochemical analysis of several normal and transformed tissues [2,3] could be due to undetectable amounts or to a genuine lack of expression of cytovillin in vivo in these tissues.

From the metabolic pulse-chase radioisotope experiments, cytovillin was very stable in vitro, with a half-life of about 25 hours. In pulse-chase experiments, no cytovillin

could be detected in immunoprecipitates of culture media (unpublished experiments), suggesting that no secretion occurs. The results further indicate that the biosynthetic rate of cytovillin should be very low in the JEG-3 cells, and this was consistent with the low cytoplasmic fluorescence observed. The metabolic labelling experiment also showed that cytovillin was synthesized as a single polypeptide in vitro. No precursor forms or proteolytic cleavage products were detected by either immunoprecipitation or immunoblotting. Only one band was also detected in the isoelectric focusing on the purified protein, indicating that cytovillin has no charge heterogeneity.

The expression and the restricted distribution of cytovillin in a wide variety of normal and transformed human cell lines and strains suggest that this protein should have a very important function in cells, which is associated with the cell surface extensions.

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