

# Immunolocalization of a Novel, Cytoskeleton-associated Polypeptide of $M_r$ 230,000 Daltons (p230)

V.-P. LEHTO and I. VIRTANEN

*Department of Pathology, University of Helsinki, SF-00290 Helsinki 29, Finland*

**ABSTRACT** Antibodies were raised against a cytoskeleton-associated, nonphosphorylated, 230,000-dalton bovine lens polypeptide (designated p230), and rendered monospecific by using a novel immunoaffinity technique. In immunofluorescence and electron microscopy of cultured fibroblasts, as well as of various other cells (endothelial, epithelial, lenticular, monocytes, neuroblastoma cells) and tissues (human kidney and liver), p230 was localized as a distinct subplasmalemmal layer in the peripheral cytoplasm of the cells. It constituted <0.3% of the total cellular protein in cultured fibroblasts and was not extractable with Triton X-100. In detergent-extracted cytoskeletal preparations of cultured fibroblasts, p230 remained as an elaborate peripheral network that showed a distribution distinctly different from that of the major cytoskeletal structures, stress fibers, cortical myosin, vinculin, and intermediate filaments (IF). The distribution was not dependent on the presence of intact stress fibers or microtubules, as shown by double-fluorescence microscopy of cells exposed to cytochalasin B or cultured in the presence of monensin and of cold-treated cells. Upon demecolcine-induced reorganization of intermediate filaments, however, the localization of p230 was rapidly altered to a dense plaque underneath the perinuclear aggregate of intermediate filaments. On the other hand, p230 seemed to colocalize with the detergent-resistant cell surface lamina, visualized in fluorescence microscopy with fluorochrome-coupled wheat germ agglutinin-lectin. The results suggest that p230 is part of a cell surface- and cytoskeleton-associated subplasmalemmal structure that may play an important role in cell surface-cytoskeleton interaction in various cells both *in vitro* and *in vivo*.

Nonionic detergents, such as Triton X-100, have been widely used to study the structure and composition of the cytoskeleton of cultured cells (1-4). Besides the major cytoskeletal components, intermediate filaments, microtubules and microfilaments, also a laminalike surface structure, containing some plasma membrane proteins and lipids, has been described in the resulting detergent-extracted cytoskeletal preparations of various cells (5, 6). Moreover, several polypeptides that remain associated with these cytoskeletal components have been detected, such as fimbrin (7), villin (8), vinculin (9), and  $\alpha$ -actinin (10), which play a role in microfilament-plasma membrane interaction, and synemin (11), paranemin (12), and microtubule-associated proteins (13), which seem to function in the regulation of the organization of intermediate filaments and microtubules, respectively. In this study we describe another cytoskeleton-associated polypeptide, originally detected in bovine lens tissue, that shows a  $M_r$  of 230,000 daltons. In various cultured mesenchymal and epithelial cells and in some human tissues it has a peripheral, subplasmalemmal location and

shows a distribution different from that of the major cytoskeletal components. It codistributes, however, with the detergent-resistant cell surface components in Triton X-100-extracted cells. The distribution remains unaltered in cells cultured in the presence of monensin or cytochalasin B but is affected, concomitant with redistribution of intermediate filaments, by demecolcine-treatment.

## MATERIALS AND METHODS

**Cells and Tissues:** Bovine eyes were obtained from a local slaughterhouse. Lenses were immediately removed and stored at  $-20^\circ\text{C}$ . Human tissue samples were obtained at autopsies performed for kidney transplantation. The samples were immediately frozen in isopentane, cooled with liquid nitrogen and stored at  $-70^\circ\text{C}$ .

Human embryonic fibroblasts, obtained from a local source, Potaroo kidney epithelial (PTK<sub>2</sub>) cells and Marbin-Darby canine kidney (MDCK) cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured in plastic Petri dishes in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal calf serum (FCS, Flow Laboratories, Irvine, Scotland) and antibiotics. Human neuroblastoma cells (cf. reference 14) were obtained

from Dr. K. Nilsson (Wallenberg Laboratory, University of Uppsala, Sweden). They were cultured in Minimum Essential Medium (MEM) supplemented with 10% FCS and antibiotics and were induced to differentiate with 12-O-tetradecanoyl-phorbol-13-acetate (TPA,  $10^{-7}$  M). Endothelial cells were harvested from human umbilical cords by perfusion with 0.2% collagenase-treatment and were cultured in Ham's F10 medium (Gibco Biocult, Glasgow, Scotland), supplemented with 20% pooled human AB serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and antibiotics as described previously (15). 75  $\mu$ g/ml endothelial cell growth supplement (ECGS, Collaborative Research, Cambridge, England) was used. Mononuclear leucocytes were isolated by standard techniques from the buffy coat fraction of human blood and cultured as described elsewhere (16, 17). Nonadherent cells were discarded and the adherent cells were subcultured. In 4–7 d, a distinct differentiation into macrophagelike cells was observed (17). Human amnion epithelial cells were obtained for a primary culture from human amniotic membranes separated from full-term postpartal placentas, and cultured in RPMI-medium with 10% FCS and antibiotics as described elsewhere (18). Bovine lens cells were cultured from small pieces of anterior capsule or marginal zone, which were trypsinized (0.5% Trypsin-TPCK; Worthington Biochemical Co., Freehold, NJ) for 30 min. The cells were washed and plated in petri dishes in RPMI-medium with 10% FCS. Human red blood cells were obtained from the Finnish Red Cross Blood Transfusion Service. Red blood cell ghosts were prepared as described by Steck et al. (19).

Detergent-resistant, substratum-attached cytoskeleton preparations were produced by treating subconfluent cells with 0.5% (wt/vol) Triton X-100 (BDH Chemicals, Poole, England) in 40 mM Tris-HCl buffer, pH 7.2, with freshly added 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO.) for 30 min as described earlier (20). This treatment removes most of the cellular proteins but leaves microfilaments, microtubules, and intermediate filaments intact, and, when performed at 4°C, also disrupts microtubules (20). For some experiments, human embryonic fibroblasts were exposed to demecol-

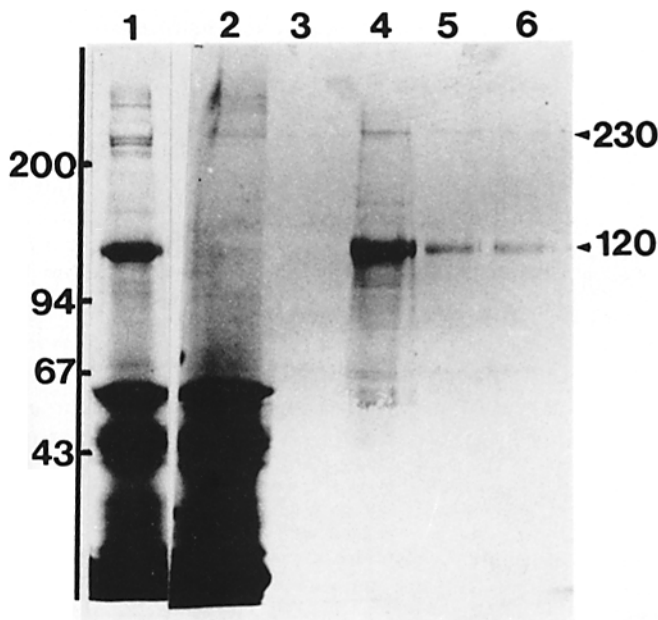


FIGURE 1 SDS PAGE analysis of the cytoskeletal preparation of bovine lens tissue and the fractions obtained from hydroxyl apatite-column chromatography during preparation of the nonvimentin 120,000- and 230,000-dalton pps of the bovine lens tissue. Coomassie Blue-staining. Lane 1, cytoskeletal preparation of bovine lens tissue shows major polypeptide bands of 230,000-, 120,000-, 58,000-, 40–43,000-, 30,000 daltons, and low  $M_r$ -material representing residual crystallins. Lane 2, run-through fractions of the urea-solubilized lens cytoskeleton preparations applied on hydroxylapatite column and washed with 6 M urea in NaCl-P buffer, 0.1% 2-mercaptoethanol, shows an enrichment of the 58,000-dalton polypeptide representing vimentin, and several smaller  $M_r$  polypeptide bands probably representing actin, degradation products of vimentin (31), and residual crystallins. Lanes 3–6, fractions eluted from the hydroxylapatite column with a linear phosphate-gradient (10 mM–200 mM) show a distinct enrichment of polypeptide bands with  $M_r$  230,000 and 120,000 daltons. The molecular weight markers (Radiochemical Centre) are indicated on the left-hand side.

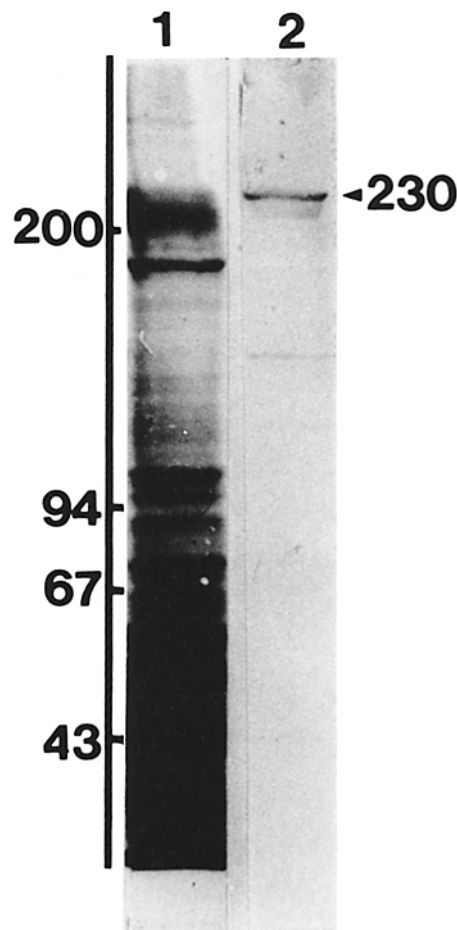


FIGURE 2 Identification of the fibroblast polypeptides reactive with the affinity-purified anti-p230. Cultured fibroblasts were harvested and electrophoresed in 8% slab gel and then transferred to nitrocellulose paper. Adjacent lanes were cut and one of them was stained with 0.1% amido black, 45% methanol, 10% acetic acid to visualize the polypeptide bands (lane 1) while the other (lane 2) was exposed to the anti-p230 and thereafter to swine anti-rabbit IgG, rabbit peroxidase/anti-peroxidase complex and DAB as described in Materials and Methods. The anti-p230 specifically reacts with a fibroblast polypeptide of a  $M_r$  of 230,000 daltons. Molecular weight markers are indicated on the left-hand side.

chine (1  $\mu$ g/ml; Colcemid, Ciba, Milan, Italy) for 12 h, to 10  $\mu$ g/ml of cytochalasin B (Sigma Chemical Co.) for 30 min or were plated and cultured in the presence of 10  $\mu$ M monensin (Eli Lilly, Indianapolis, IN) for 12–24 h as described (21).

**Production of Anti-p 230 Antibodies (Anti-p230):** Bovine eye lenses were homogenized in cold NaCl-P buffer (10 mM phosphate buffer, pH 7.2, 140 mM NaCl) supplemented with 1 mM PMSF and 0.5 mM N- $\alpha$ -tosyl-L-lysine chloromethyl ketone (TLCK; Sigma Chemical Co.). The particulate material was harvested by a low-speed centrifugation and then extracted twice with 0.5% (wt/vol) Triton X-100 in 50 mM Tris-HCl buffer, pH 7.2, 1 mM PMSF, 0.5% TLCK and then with low- and high-ionic, actomyosin-extracting buffers as described earlier (20). The cytoskeletal preparation was dissolved in 8 M urea in 50 mM Tris-HCl buffer, pH 7.3, 5 mM  $MgCl_2$ , 5 mM dithiothreitol for 4 h on ice. Undissolved material was removed by centrifugation. The supernatant was dialyzed against 6 M urea in NaCl-P buffer, 0.1% 2-mercaptoethanol and then applied on a hydroxylapatite column (HA-Ultrogel, LKB, Bromma, Sweden), equilibrated with the same buffer. The column was thoroughly washed with the same buffer, and the attached material was eluted using a linear phosphate-gradient (10 mM–200 mM). The fractions containing the cytoskeletal polypeptides of 120,000 and 230,000 daltons (see Fig. 1) were pooled, dialyzed against water, and lyophilized. 1 mg of the lyophilized material in 0.5 ml of NaCl-P buffer was dissolved in 0.5 ml of Freund's complete adjuvant and injected i.d. at multiple sites in a rabbit. A booster injection was given 4 wk later, and the rabbit was bled 7 d after the booster. Affinity purification of the anti-p230 was performed by incubating the crude hyperimmune serum with nitrocellulose strips

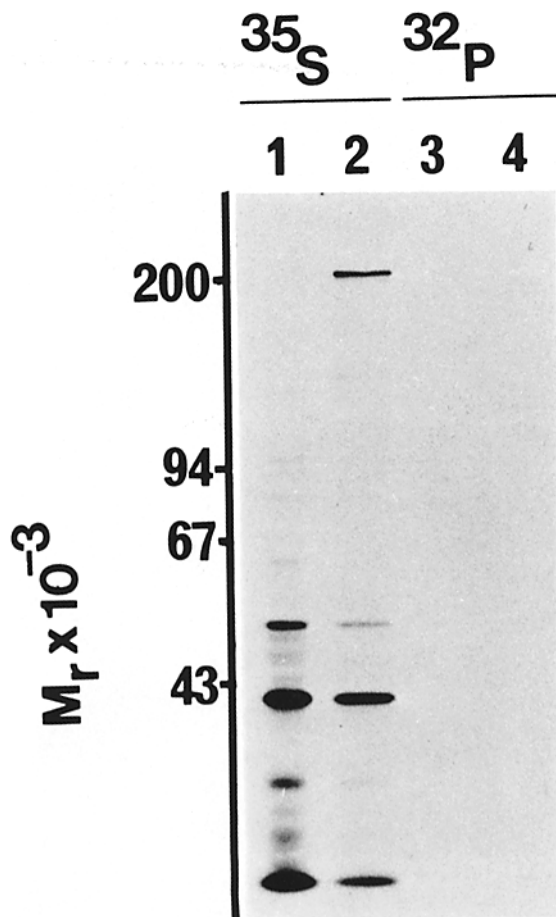


FIGURE 3 SDS PAGE analysis and fluorography of the anti-p230-immunoprecipitates of cultured human fibroblasts. Cultured human fibroblasts were labeled with [ $^{35}\text{S}$ ]methionine (lanes 1 and 2) or 32-phosphorus (lanes 3 and 4), extracted, and immunoprecipitated either with nonimmune serum (lanes 1 and 3) or with anti-p230 (lanes 2 and 4) as described in Materials and Methods. Lane 1 shows five low molecular weight polypeptides which were precipitated nonspecifically with the nonimmune serum. The same polypeptides are discernible in lane 2 in which also the specific precipitation of a 230,000-dalton-band is seen. No phosphorylated polypeptides could be detected in the immunoprecipitates as seen in lane 4. Molecular weight markers are indicated on the left-hand side.

containing the p230-bands, electrophoretically transferred from the polyacrylamide gel (see below). The strips made an affinity matrix containing ~100–300  $\mu\text{g}$  of the antigen when transferred from two overloaded polyacrylamide gels and contained only the p230-doublet as judged by reelectrophoresis. Before mixing with the antiserum, the strips were soaked in NaCl-P buffer, 3% bovine serum albumin (BSA) to prevent nonspecific binding of serum proteins. After incubation with the hyperimmune serum, the strips were extensively washed with NaCl-P buffer, and the bound antibodies were eluted with 0.2 M glycine, pH 2.8, neutralized with sodium hydroxide, and dialyzed against NaCl-P buffer.

**Metabolic Labeling and Immunoprecipitation:** For metabolic labeling, subconfluent cultures of human fibroblasts were kept for 2 h in methionine-depleted RPMI 1640 medium without fetal calf serum. Thereafter, 20  $\mu\text{Ci}/\text{ml}$  of L-[ $^{35}\text{S}$ ]methionine (600 Ci/mmol, Radiochemical Centre, Amersham, England) was added and the cells were kept in culture for an additional 24 h. For metabolic labeling with radioactive phosphorus, the cells were cultured in phosphate-free MEM, supplemented with 100  $\mu\text{Ci}/\text{ml}$  of phosphorus-32 (Radiochemical Centre) for 12 h.

For immunoprecipitation experiments, metabolically labeled cells from one 90-mm culture dish were dissolved in 70  $\mu\text{l}$  of 0.1% (wt/vol) sodium dodecyl sulphate (SDS; Merck, Darmstadt, FRG) in 50 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl. Undissolved material was removed by centrifugation and the supernatant was heated at 95°C for 3 min after which 10% (wt/vol) Triton X-100 and 10% (wt/vol) sodiumdesoxycholate (DOC; Merck) were added to a final concen-

tration of 1%. Two  $\mu\text{l}$  of nonimmune rabbit serum (10 mg/ml) was added to the supernatant for 10 min followed by addition of 60  $\mu\text{l}$  (wt/vol) of 10% solution of washed Protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden) in 50 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl. After centrifugation, the supernatant was collected and divided in two. 1  $\mu\text{l}$  of nonimmune rabbit serum (10 mg/ml) was added to one portion, and 1  $\mu\text{l}$  of the anti-p230 (10 mg/ml) to the other. After incubation overnight at 4°C, Protein A-Sepharose beads were added as described above for 30 min. The antigen-antibody-protein A-Sepharose-complexes were harvested by centrifugation and washed twice with 50 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl, twice with 50 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% DOC and twice with 50 mM Tris-HCl buffer, pH 7.2, before analysis in PAGE.

**Electrophoretic Techniques and Identification of the Immunoreactive Polypeptides with the Anti-p230:** PAGE in the presence of SDS was performed on 6.5% or 8% slab gels with 3% stacking gel according to Laemmli (22). Densitometric analyses of the electrophoretically separated polypeptides were performed using a Joyce-Loebl Chromoscan-densitometer (Joyce & Loebl Co. Ltd., Gateshead, England). For fluorography, the gels were processed according to Laskey and Mills (23). Dried gels were autoradiographed on Kodak X-Omat film, and for  $^{32}\text{P}$ -autoradiography intensifying screens were used.

Transfer of electrophoretically separated polypeptides onto a nitrocellulose paper was carried out in 25 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, 20% (vol/vol) methanol, using a commercial blotting apparatus (Trans-Blot, Bio-Rad Laboratories, Richmond, CA) according to the method of Towbin et al. (24). For immunoblotting, the nitrocellulose sheets were soaked in NaCl-P buffer, 3% BSA, 10% normal swine serum (NSWS) for 120 min, washed and then exposed to 50  $\mu\text{g}/\text{ml}$  of the anti-p230 in NaCl-P buffer 3% BSA, 10% NSWS for 6 h. After washing, the nitrocellulose sheets were incubated with 50  $\mu\text{g}/\text{ml}$  of swine anti-rabbit IgG (DAKO Immunochemicals, Copenhagen, Denmark) for 60 min, washed and then exposed to 25  $\mu\text{g}/\text{ml}$  of peroxidase/rabbit anti-peroxidase complex (DAKO). After 30 min incubation, 3,3'-diamino-benzidine-tetrahydrochlorid (DAB; Fluka AG, Buchs, Switzerland) in 10 mM Tris-HCl buffer, pH 7.5, 0.05%  $\text{H}_2\text{O}_2$ , was added to develop the peroxidase reaction.

**Immunofluorescence Microscopy:** For indirect immunofluorescence (IIF) microscopy, whole cells were fixed either in 2.5% (wt/vol) paraformaldehyde in NaCl-P buffer (PF-fixation), in  $-20^\circ\text{C}$  methanol for 5 min, or in paraformaldehyde as described above but followed by treatment with 0.01% Nonidet-40 (NP-40; BDH Chemicals) (PF-NP-fixation) to permeabilize the cells (25). Triton X-100-extracted cytoskeletal preparations were fixed in paraformaldehyde. For a study of suspended cells in IIF, trypsinized (0.25% Trypsin TPKC) cells were allowed to attach onto coverslips coated with poly-L-lysine (Type II, Sigma Chemical Co.) and then fixed with paraformaldehyde and permeabilized with NP-40. Cryostat sections of deep frozen specimens of human tissues were fixed in  $-20^\circ\text{C}$  methanol for 5 min. After fixation, the specimens were reacted with the primary antiserum and then exposed to fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isothiocyanate (TRITC)-conjugated second antibody (usually goat anti-rabbit IgG antiserum, Cappel Laboratories Inc., Cochranville, PA). Control experiments employing rabbit preimmune serum or anti-p230 antiserum preabsorbed with purified p230 gave negative staining results on all cells and tissues studied.

Rabbit antivimentin antibodies have been characterized elsewhere (18). Vinculin was isolated from chicken gizzard as described by Feramisco and Burridge (26) and antivinculin antibodies were raised in rabbits as described elsewhere

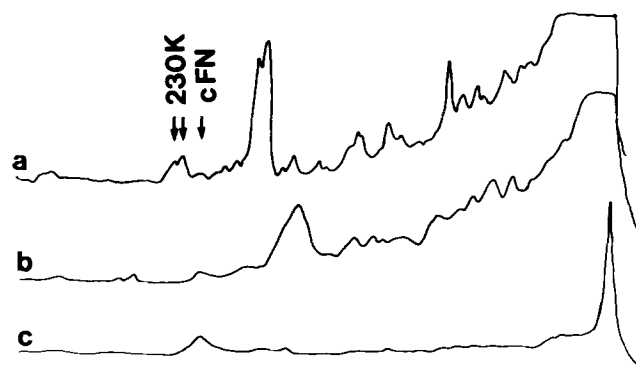


FIGURE 4 Quantitative densitometric scanning of Coomassie Blue-stained SDS PAGE gels of Triton X-100 nonextractable material (a) and of the first Triton X-100-supernatant (b) of cultured human fibroblasts. In (c) cellular fibronectin ( $M_r$  220,000 daltons) was run as a marker molecule.

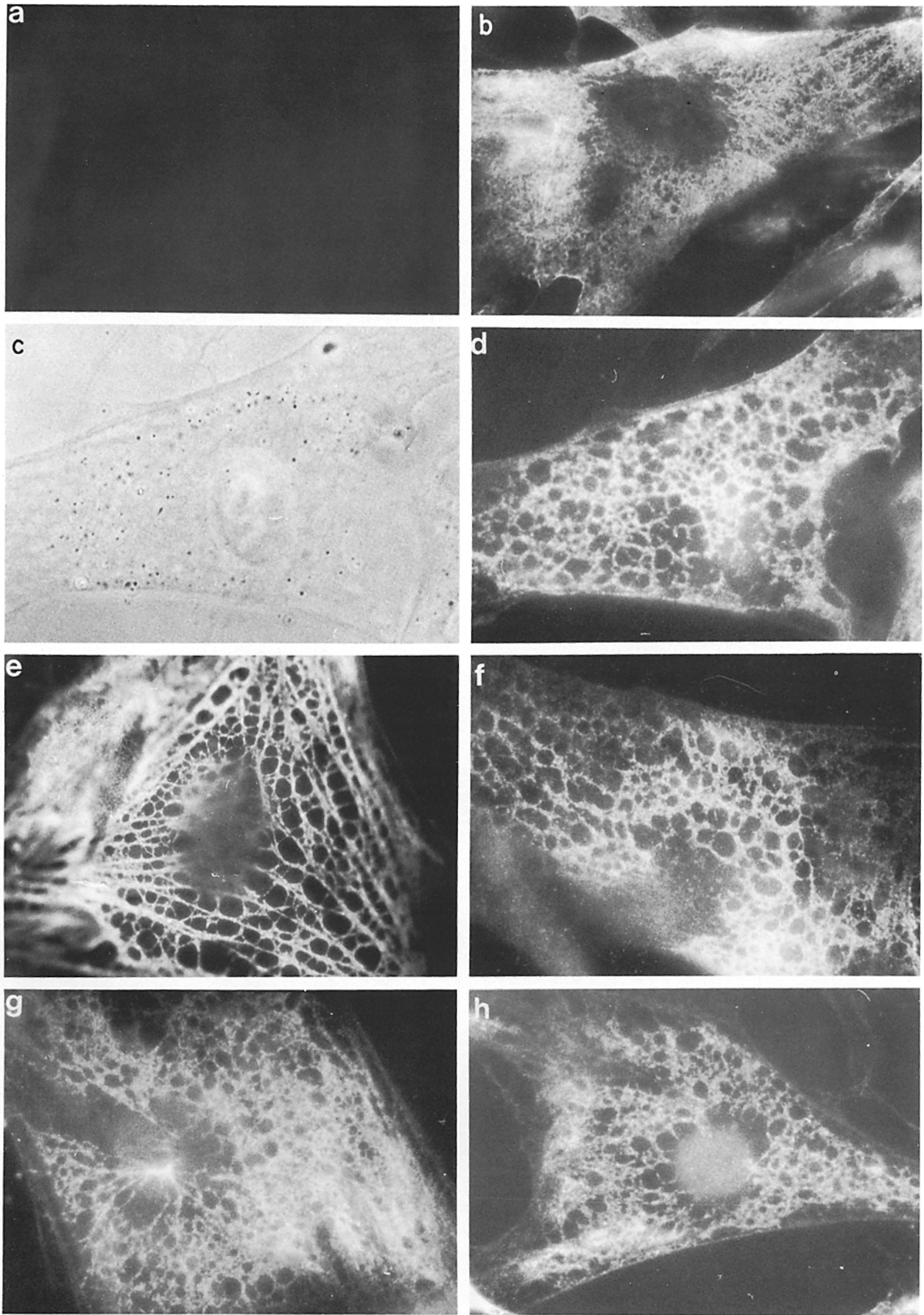


FIGURE 5 Fluorescence micrographs after staining with anti-p230 and FITC-anti-rabbit IgG. Cultured human fibroblasts fixed with paraformaldehyde (a) or methanol (b) and cytoskeletal preparations (produced at 20°C) of the same cells fixed with paraformaldehyde (c-h). c, A phase contrast micrograph of the same field as in d.

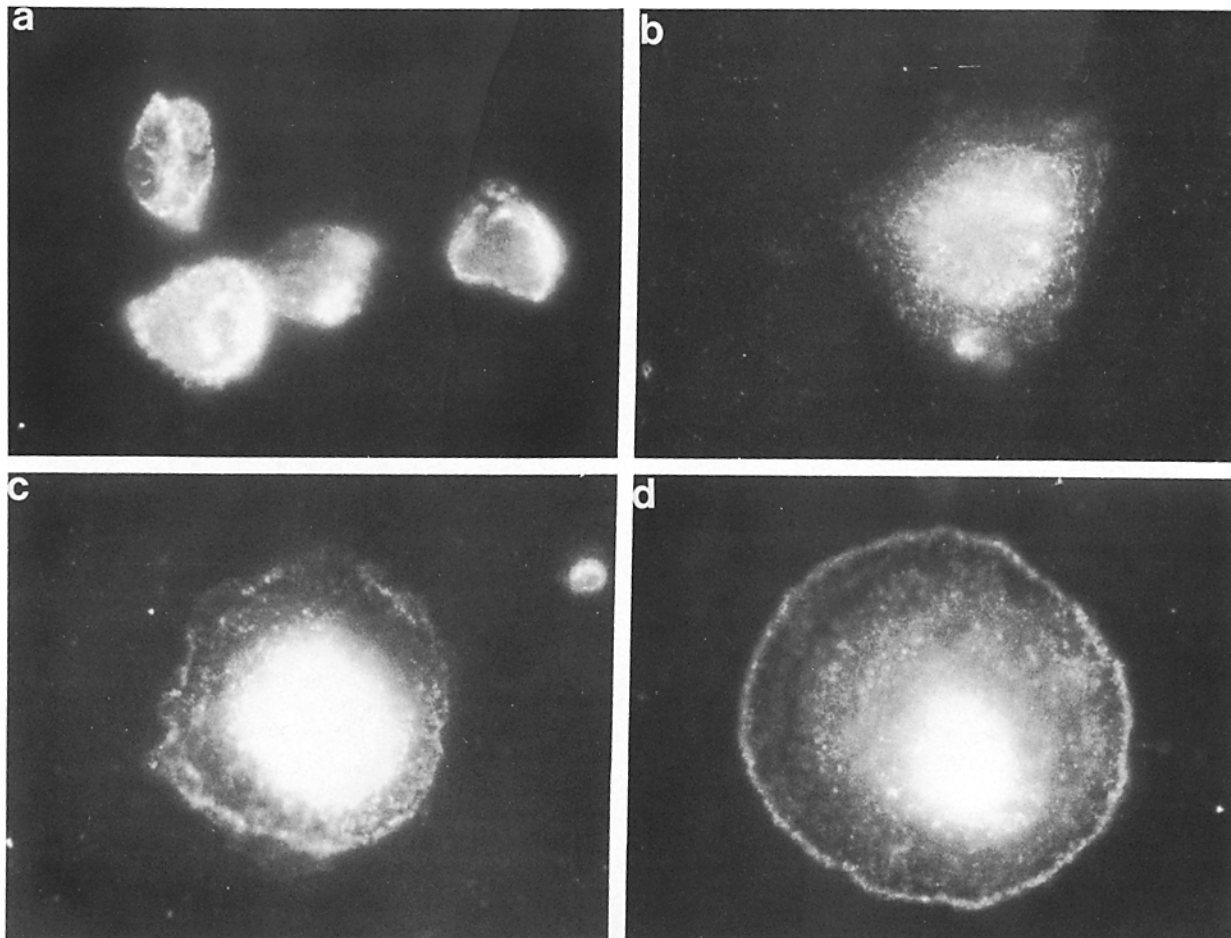


FIGURE 6 Fluorescence micrographs after staining with anti-p230 and FITC-anti-rabbit IgG. Suspended human fibroblasts attached to poly-L-lysine-coated coverslips (a) and spreading cells 1 h (b), 2.5 h (b), and 4 h (c) after attachment. The cells were fixed with paraformaldehyde and permeabilized with NP-40 before staining.

(17). Affinity-purified rabbit antiactin and antimyosin antibodies (27) were a kind gift from R. A. Badley (Unilever Research, Sharnbrook, England). For double-labeling experiments, also monoclonal human antivimentin antibodies (18) were used. For visualization of filamentous (F) actin, NBD-phalloidin (Molecular Probes Inc., Plano, TX) (28) was used in some experiments. The surface lamina of the cytoskeletal preparations of cultured fibroblasts was visualized by using TRITC-conjugated wheat germ agglutinin lectin (WGA) (Vector Laboratories, Burlingame, CA) as described in detail elsewhere (29). The specimens were studied in a Zeiss Universal microscope equipped with an epilluminator III RS, phase contrast optics and filters for FITC- and TRITC-fluorescence.

**Immunoelectron Microscopy (IEM):** For IEM, subconfluent fibroblasts, cultured in petri dishes, were fixed in 0.1% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, containing 0.05% saponin (BDH) for 30 min. Thereafter the cultures were exposed to the anti-p230p in the presence of 1% normal sheep serum, 3% BSA and 0.05% saponin. Subsequently, the specimens were exposed to biotinylated goat anti-rabbit IgG, avidin-biotinyl-peroxidase complex (Vectastain, Vector Laboratories) and DAB in 50 mM Tris-HCl, pH 7.6 with freshly added  $H_2O_2$ . Controls which included rabbit preimmune serum as a primary antiserum and preabsorption of anti-p230 with purified p230 gave negative staining results. The cells were postfixated in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Horizontal and vertical sections were made and, either unstained or after staining with uranyl acetate and lead citrate, examined in a Jeol 100CX electron microscope at the Department of Electron Microscopy, University of Helsinki.

## RESULTS

### Production and Characterization of Anti-p230

In SDS PAGE analysis of cytoskeletal preparations of lens tissue only a few major polypeptides were present (Fig. 1, lane

1). The most prominent polypeptides had  $M_r$  of 230,000 (a doublet), 120,000, 58,000, and 40–45,000 daltons, the latter two being identified as vimentin and actin, respectively, in numerous studies (for a review, see reference 30). Moreover, there was also low  $M_r$  material which represents residual crystallins (30). To further purify the 230,000 and 120,000-dalton polypeptides, we took the advantage of the fact that vimentin can be removed from lens cytoskeletal material using hydroxylapatite (HA) column chromatography (31). Electrophoretic analysis of the HA-column eluate indicated that the 230,000- and 120,000-dalton-proteins could be eluted without vimentin if thoroughly preextracted cytoskeletal lens preparations were used as a starting material (Fig. 1, lanes 2–6). The eluted 230,000-dalton protein seemed to represent the higher  $M_r$  band of the 230,000-dalton pp doublet seen in unfractionated lens cytoskeletal material (Fig. 1, lane 1).

IIF-microscopy with the crude hyperimmune serum raised against the 120,000- and 230,000-dalton protein-containing HA-fractions showed positive staining reaction in both cultured fibroblasts and lens tissue sections. In immunoblotting, the antiserum reacted with lens cytoskeletal polypeptides of  $M_r$  120,000 and 230,000 daltons while in fibroblasts only a reaction with a 230,000-dalton band was seen (not shown). In order to affinity-purify the antibodies reacting with the 230,000-dalton polypeptides, present in both lens tissue and fibroblasts, we incubated and eluted the crude hyperimmune serum with electrophoretically separated 230,000-dalton bands fixed in



nitrocellulose paper as described in Materials and Methods. Fig. 2 shows that the affinity-purified anti-p230 reacted with only a single polypeptide of  $M_r$  230,000 daltons on the nitrocellulose blot of electrophoretically separated polypeptides of cultured fibroblasts (Fig. 2). The monospecificity of the anti-p230 was demonstrated also by immunoprecipitation in which a specific reaction was only seen with a polypeptide of  $M_r$  230,000 daltons (Fig. 3, lanes 1 and 2). Parallel immunoprecipitation with  $^{32}\text{P}$ -labeled cells showed that the precipitated 230,000-dalton polypeptide is not phosphorylated under normal culture conditions (Fig. 3, lanes 3 and 4).

#### Triton X-100-Extractability of p230

To study the detergent-resistance of p230, we harvested confluent cells from one culture dish by trypsinization and then extracted them twice with a small volume of Triton X-100 as described above. The extracts were ultracentrifuged and the resulting supernatants and the nonextractable pellet were run in SDS PAGE. The gel was stained with Coomassie Brilliant Blue and the lanes were scanned in a Joyce-Loebl densitometer. Fig. 4 shows that p230 remains in the Triton-nonextractable material. The doublet constitutes <0.3% of the total cellular protein. Fig. 4 also shows that the Triton-extractable polypeptide showing a slightly lower  $M_r$  than p230 probably represents cytoplasmic cellular fibronectin as judged from its coelectrophoresis with purified cellular fibronectin (BRL, Bethesda, MA) (Fig. 4).

#### IF Findings

**-ANTI-P230 IN CULTURED HUMAN FIBROBLASTS:** Anti-p230 did not show reaction with intact cultured fibroblasts fixed with paraformaldehyde (Fig. 5a), indicating that p230 is not exposed at the cell surface, while in cells fixed with methanol a finely reticular, surface-associated fluorescence was seen (Fig. 5b). A surface-associated, yet somewhat more distinctly meshworklike reticular cytoplasmic staining was also seen in cells fixed with paraformaldehyde and subsequently permeabilized with NP-40. A similar, strong reaction was seen in Triton-cytoskeletons of the cells in which the antiserum decorated a subplasmalemmal, laminalike reticulum which seemed to correspond to the whole substratum attachment area of the cell and which also bridged the nucleus as a domelike structure (Figs. 5c-h). Interestingly, in some cells p230-reticulum was lacking around the nuclear area and in these cells short fiberlike formations seemed to connect the network to the substratum-facing aspect of the nucleus (Fig. 5e, g, and h).

No structural entities were revealed with the anti-p230 in suspended cells which were permeabilized with NP-40 after fixation with paraformaldehyde (25) (Fig. 6a). Soon after initial spreading, a bright, for the most part subnuclear fluorescence could be revealed in the cells (Fig. 6b and c). Later on, the fluorescence could be confined to both the ventral surface and the supranuclear dome of the cell. Moreover, a distinctly ringlike fluorescence could be discerned along the cell periphery at the ventral surface of the cells (Fig. 6d).

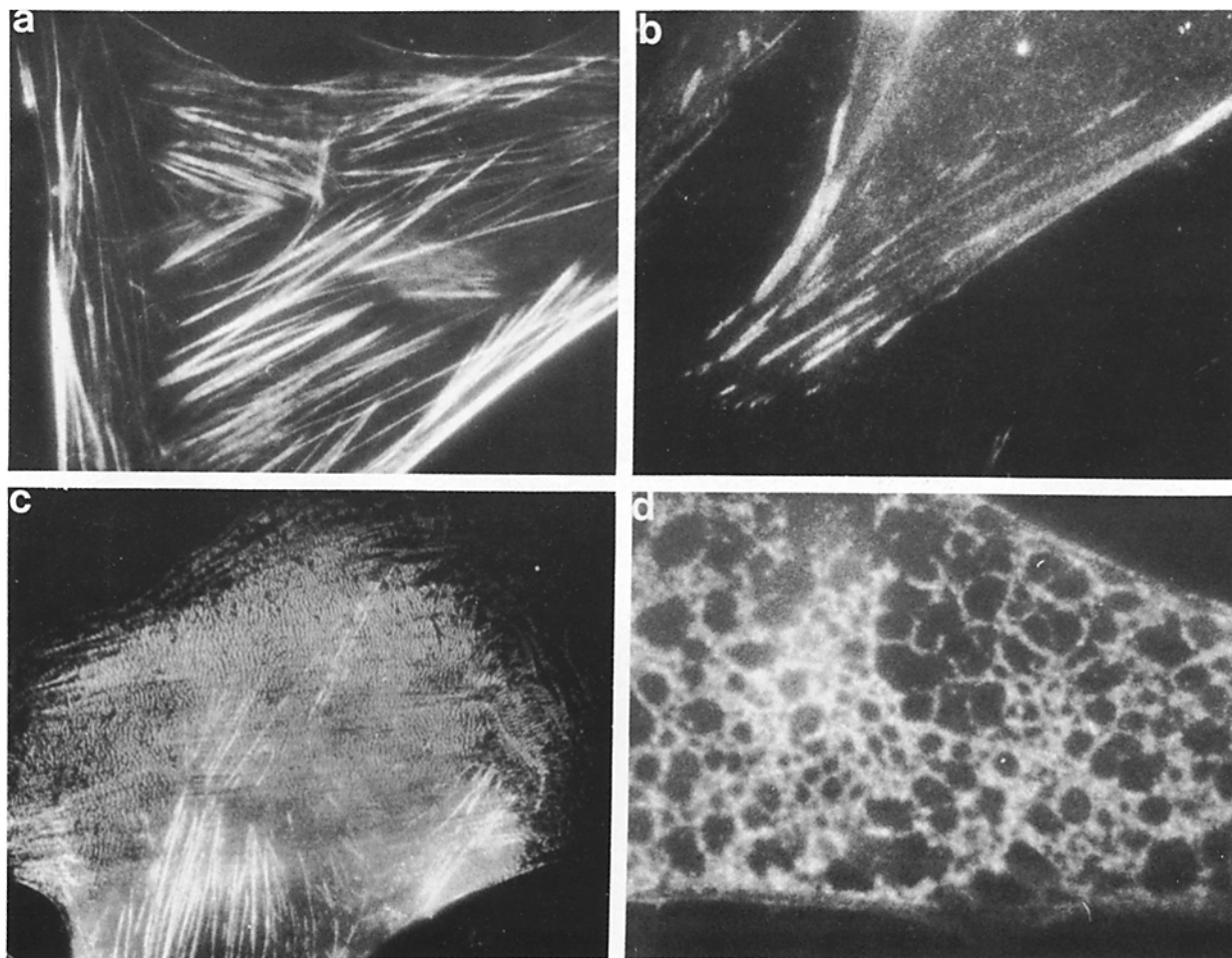


FIGURE 7 Fluorescence micrographs of Triton X-100-cytoskeletal preparations of cultured human fibroblasts fixed with paraformaldehyde and stained with antiactin antiserum (a), antivinculin antiserum (b), antimyosin antiserum (c), and anti-p230 (d).

**-P230 AND CYTOSKELETAL STRUCTURES:** The fluorescence pattern obtained with the anti-p230 (Fig. 7 *d*) was clearly different from the distribution of the other major subplasmalemmal cytoskeletal polypeptides actin, vinculin, and myosin, actin being localized to stress fiberlike structures (Fig. 7 *a*), vinculin to surface-associated plaques (Fig. 7 *b*), and myosin to both stress fibers and to an elaborate, surface-associated lamina (Fig. 7 *c*).

No difference in p230-distribution could be seen if the cells were extracted either in the microtubule-depolymerizing conditions (cold treatment, reference 32) or at 20°C to preserve the microtubular organization (compare Figs. 5 and 8). However, if the cultured cells were pretreated with demecolcine for 12 h, to disrupt the microtubular system and to induce perinuclear coiling of intermediate filaments (33), a distinct reorganization of the fluorescence pattern of p230 could be seen (Fig. 8 *a* and *c*). In double-IF microscopy of the demecolcine-treated cells, the anti-p230-fluorescence could be seen to be confined almost exclusively underneath the perinuclear aggregate of intermediate filaments (Fig. 8 *c* and *d*).

Fig. 9 shows p230- and filamentous actin in fibroblasts cultured for 12 h in the presence of monensin. The cells lack actin-containing stress fibers (Fig. 9 *a*), as shown also previously (21), but display the p230-pattern indistinguishable from that of control cells in both PF-NP-fixed whole cells and Triton X-100-extracted cytoskeletal preparations (Fig. 9 *b* and *c*). Also the cytochalasin B-treated, PF-NP-fixed whole cells (Fig. 9 *d* and *e*) and Triton X-100-extracted cytoskeletal preparations

(Fig. 9 *f*) retained normal p230-distribution (Fig. 9 *e* and *f*) although the microfilament-organization was totally disrupted (Fig. 9 *d*).

**-P230 AND THE DETERGENT-RESISTANT SURFACE LAMINA:** Fig. 10 shows double-staining with TRITC-WGA, decorating the detergent-resistant cell surface domains which carry externally disposed sialic acid residues (29), and with anti-p230. Both stainings show a similar location (Fig. 10 *a* and *b*) although usually a weaker reaction was seen with anti-p230 at cell edges. An extensive and almost complete codistribution was seen, however, in central areas and especially at the ventral surface of the cells (Fig. 10 *c* and *d*). When focused at the dorsal surface of the cells, a more diffuse staining was seen, showing, however, a clear codistribution of the fluorescence patterns (Fig. 10 *e* and *f*).

**-P230 IN OTHER CULTURED CELLS:** Fig. 11 demonstrates the presence of p230 in endothelial cells (*a*), in monocyte-derived macrophages (*c*), in uninduced neuroblastoma cells (*e*), in TPA-induced neuroblastoma cells (*f*) and Fig. 12 in human amnion epithelial cells (*a*), in PTK<sub>2</sub>-cells (*b*), in MDCK-cells (*c*), and in bovine lens cells (*d*). In all cells, p230 showed a peripheral, cortical distribution. In endothelial cells, epithelial cells and in lens cells there was also a distinct, circumferential condensation of p230 at the ventral surface of the cells. In epithelial and lens cells, a similar condensation was also seen at collision sites between adjacent cells. Fig. 11 *c* and *d* demonstrate the differential organization of p230 and actin also in monocyte-derived macrophages.

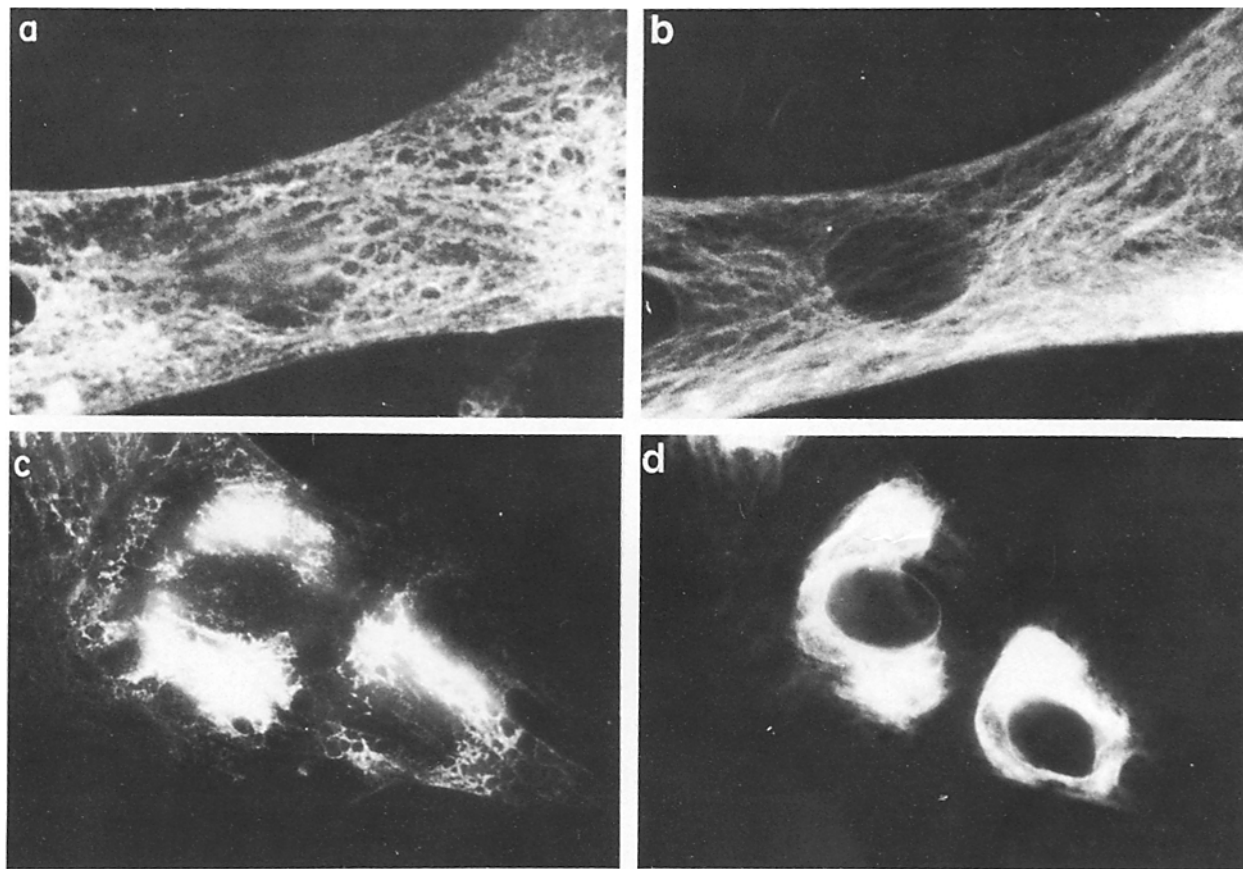


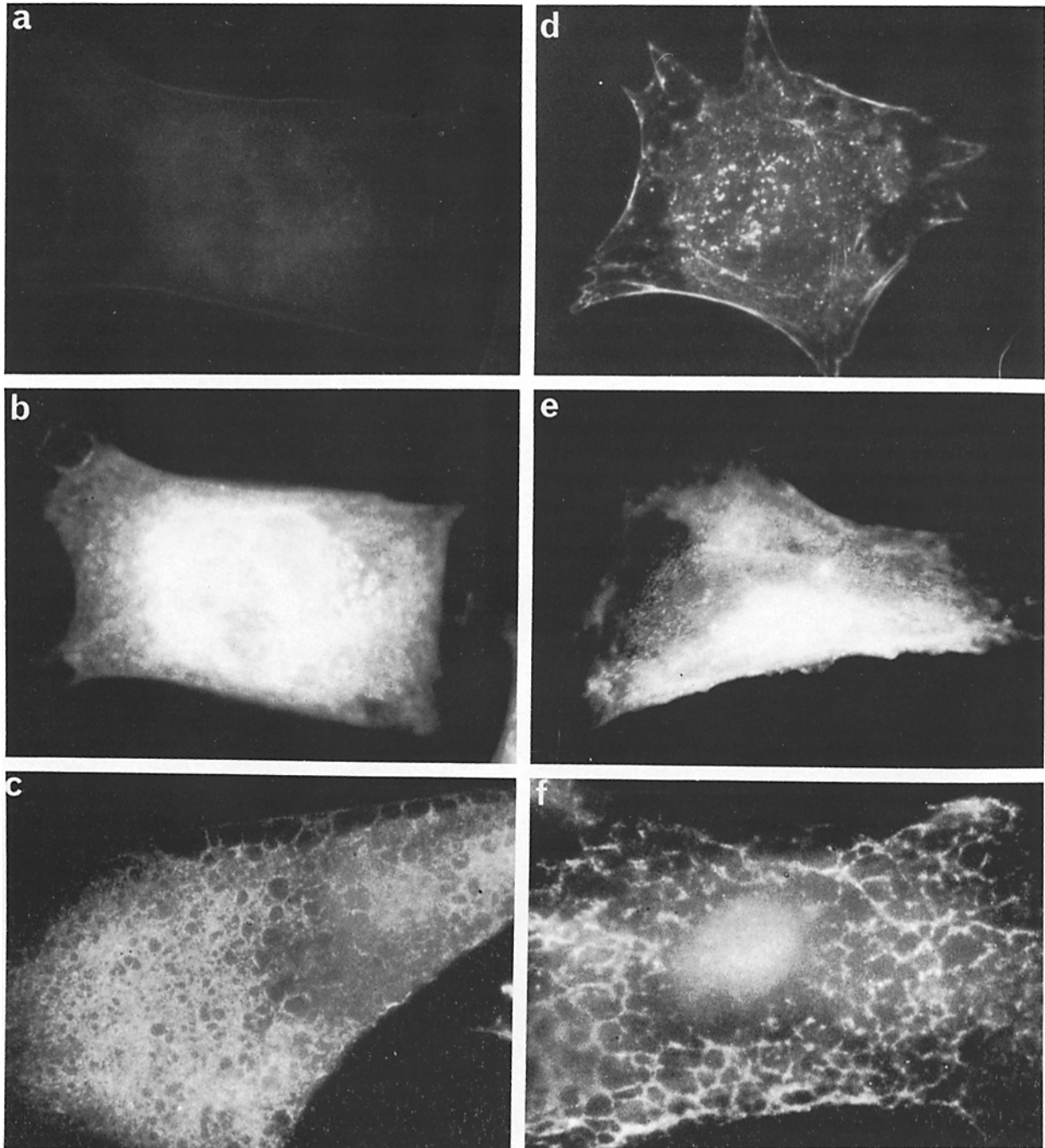
FIGURE 8 Fluorescence micrographs of Triton X-100-cytoskeletons of cultured human fibroblasts after double-staining with anti-p230 (*a* and *c*) and antivimentin antiserum (*b* and *d*). The cytoskeletal preparations in (*a*) and (*b*) were produced at 4°C to disrupt microtubules, and the cells in *c* and *d* were pretreated with demecolcine before the staining procedure.

**-P230 IN HUMAN TISSUES:** At tissue level, the distribution of p230 was studied in human liver and kidney. In liver parenchyme, p230 was seen in hepatocytes in which it showed a distinctly circumferential organization in the periphery of the cells (Fig. 13 *a*). In the bile duct epithelium, p230 was seen as a peripheral band, as well (Fig. 13 *b*). In the vascular bed, p230 was seen in endothelial cells but not in the muscle cells of the vessel walls (Fig. 13 *c*). In kidney, p230 was found in tubular epithelium (Fig. 13 *d* and *e*) and throughout the glomeruli (Fig. 13 *f*). In tubuli, the staining pattern was variable in that in

some parts a circumferential staining was seen while in other parts a distinctly basal staining pattern was seen.

#### *Immunoelectron Microscopy (IEM)*

In IEM, p230 was localized as a narrow, dense rim at the cytoplasmic face of the plasma membrane in GA-saponin-fixed fibroblasts (Fig. 14 *a-d*). Often, dense accumulations of cytoplasmic filaments were seen associated with p230-condensations (Fig. 14 *b* and *c*). No decoration of the outer cell surface could be seen.



**FIGURE 9** Fluorescence micrographs of fibroblasts after staining with NBD-phalloidin (*a* and *d*) or with anti-p230 (*b*, *c*, *e*, and *f*). The cells were cultured for 12 h in the presence of monensin (*a-c*) or for 30 min in the presence of cytochalasin B (*d-f*). *a*, *b*, *d*, and *e* are whole cells fixed with paraformaldehyde and permeabilized with NP-40, *c*, and *f*, are Triton X-100-extracted cells fixed with paraformaldehyde.



### Detection of an Immunoanalogue of p230 in Human Red Blood Cells

Transfer of electrophoretically separated polypeptides of Triton HX-100-extracted human red blood cell ghosts to nitrocellulose and a subsequent exposure to anti-p230 revealed a cross-reacting polypeptide of  $M_r$  230,000 daltons (Fig. 15). The detergent resistance and electrophoretic mobility strongly suggest that this 230,000-dalton protein represents  $\alpha$ -spectrin (cf. reference 34).

### DISCUSSION

The role of cytoskeleton as the organizational framework of the cell has been elucidated in detail in human red blood cells (35). This has been achieved to a great extent by applying nonionic detergent-treatments which tend to preserve protein-protein interactions (36), known to be important in the maintenance of the cytoskeletal architecture of the cells (35). With the same kind of approach, the major cytoskeletal components of cultured adherent cells have been revealed, as well (1, 4, 20).

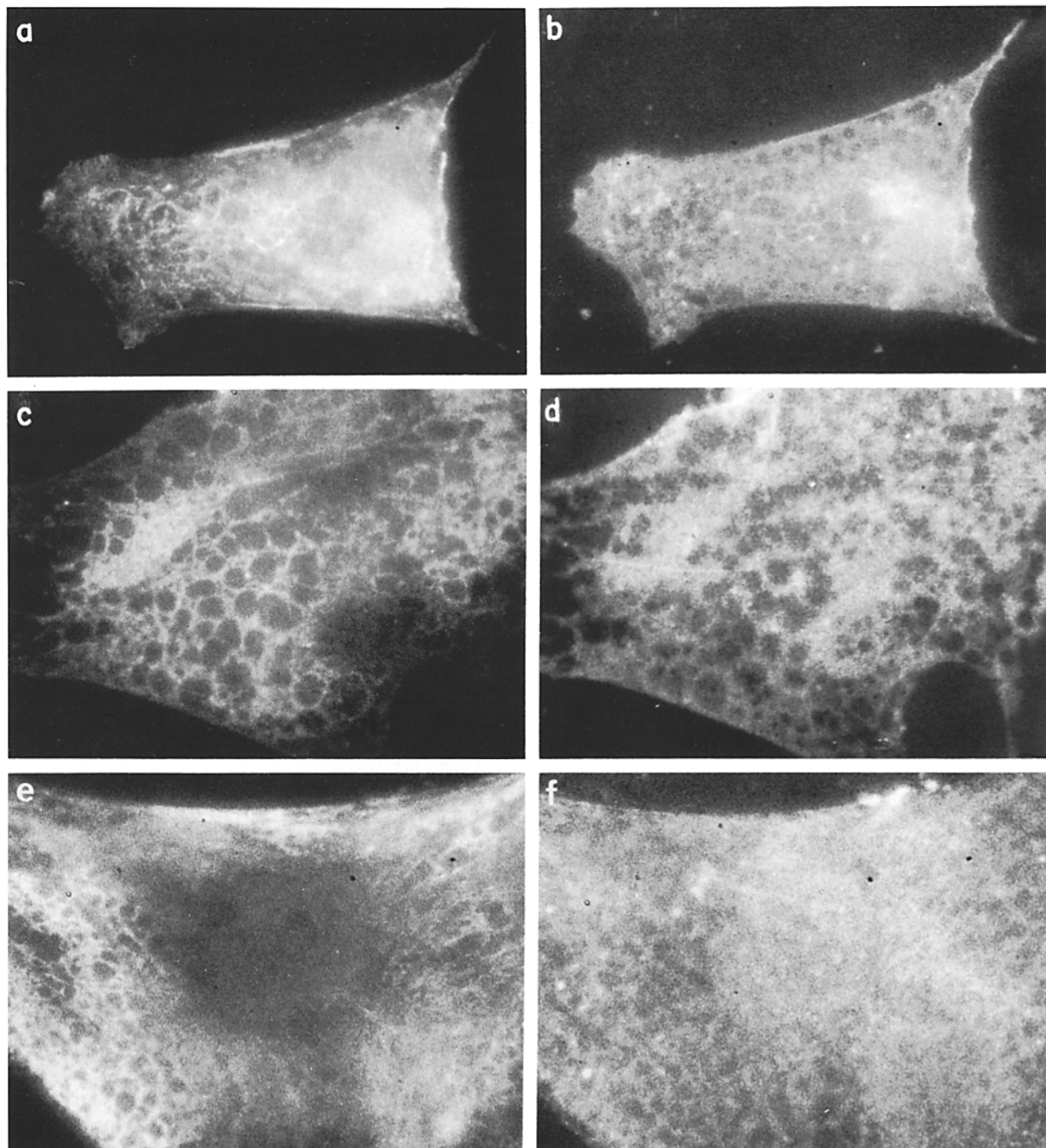


FIGURE 10 Fluorescence micrographs of cytoskeletal preparations of cultured fibroblasts after double-staining with anti-p230, FITC-anti-rabbit IgG (*a*, *c*, and *e*) and TRITC-WGA (*b*, *d*, and *f*). In *c* and *d* the focus is at the growth substratum and in *e* and *f* at the dorsal surface of the cytoskeletal preparations.

Such studies have also shown that cytoskeletal structures interact with each other and with plasma membranes and have an influence on cell surface phenomena (6, 37-41). The molecular mechanisms of these interactions have remained, however, still largely unelucidated.

In this study we describe a novel polypeptide of  $M_r$  230,000 daltons that resists Triton X-100-treatment and remains in the cytoskeletal preparations of cultured human fibroblasts. Immunolocalization studies on various unextracted cells, cytoskeletal preparations and tissue sections indicated that p230

has a peripheral localization at the cytoplasmic aspect of the plasma membrane. It appeared to distribute homogeneously throughout the cellular domain although in many cultured fibroblasts also p230-free areas were found, especially in a subnuclear location. On the other hand, in some epithelial cells, in endothelial cells, and especially in monocyte-derived macrophages, a prominent, plate- or ring-like organization was seen at the ventral surface. The well-preserved organization of the other cytoskeletal structures indicates that the detergent-treatment does not lead to deterioration or collapse of the

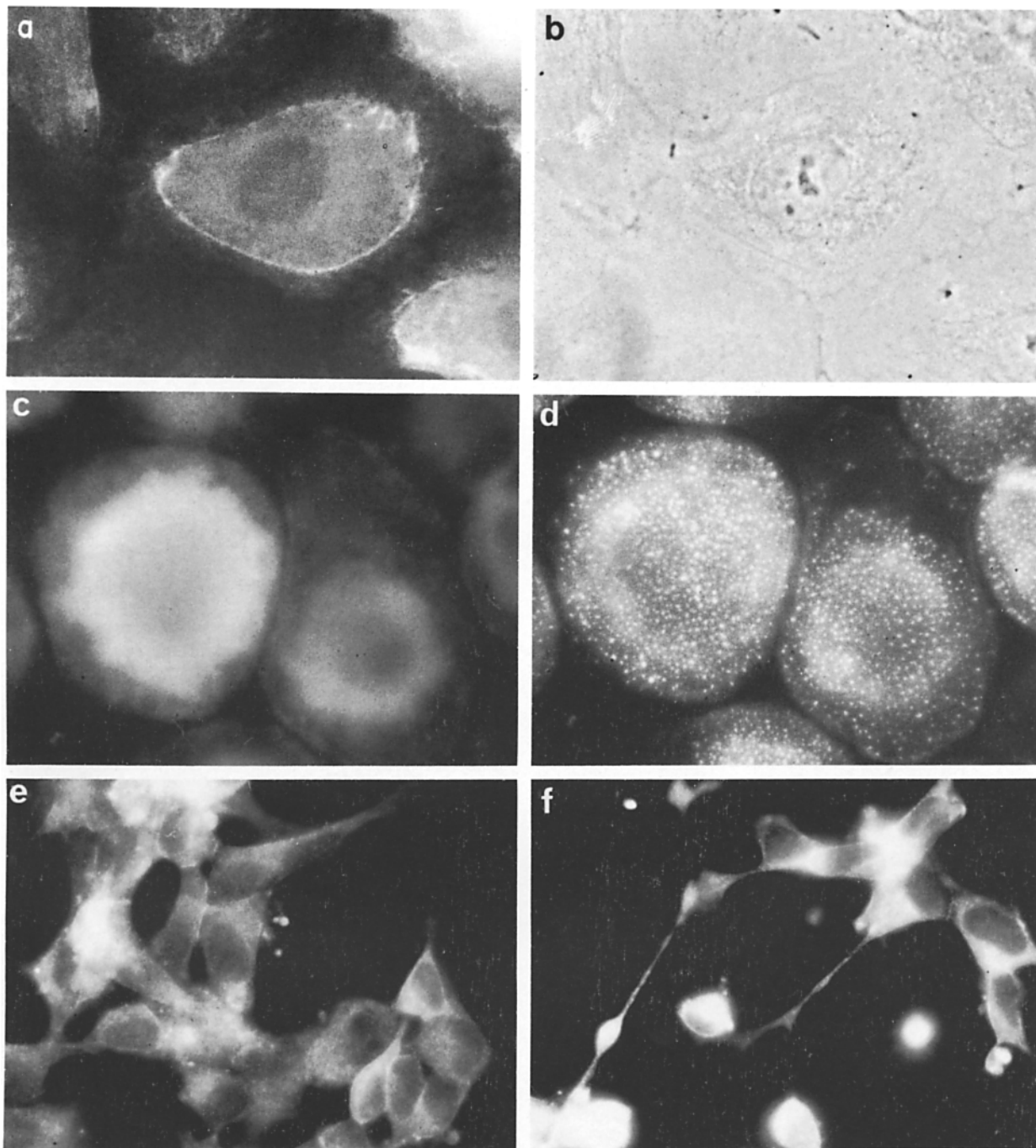


FIGURE 11 Fluorescence micrographs of umbilical cord endothelial cells (a), monocyte-derived macrophages (c), uninduced human neuroblastoma cells (e), TPA-induced human neuroblastoma cells (f) after staining with anti-p230 and FITC-anti-rabbit IgG. b, A phase contrast micrograph of the same field as in a, and d represents antiactin staining of the same field as in c. PF-NP-fixation.

cytoskeletal architecture, ruling out the possibility of artefactual association of p230 with neighboring structures. P230 did not show codistribution with the major detergent-resistant, cytoskeletal structures. In this respect it differs from such cytoskeleton-associated proteins as synemin (11) and paranemin (12), which show a complete codistribution with intermediate filaments. Cold-treatment, which disrupts microtubules but does not affect intermediate filaments (32), did not seem to have any effect on the distribution of p230. Besides fibroblasts, the differential distribution of actin and p230 was also seen in monocyte-derived macrophage. Moreover, in cells cultured in the presence of monensin and in cytochalasin B-treated cells, both of which lack actin-containing stress fibers (21), p230 displayed an unaltered distribution. On the other hand, the p230-network responded strikingly to demecolcine-treatment which causes depolymerization of microtubules and brings about perinuclear coiling of intermediate filaments (33). These results suggest that p230-organization is not directly dependent on microfilament- or microtubular organization but may be, in some way, interlinked with intermediate filaments. The mechanism of this possible linkage is, however, unclear. We have recently presented evidence for interaction between plasma membrane- and intermediate filaments in cultured fibroblasts, and in some other cell types, such as chicken red blood cells and bovine eye lens cells, vimentin-type intermediate filaments have been convincingly shown to interact with plasma membrane or with subplasmalemmal material (42-44), making a direct contact between these filaments and p230 a feasible

possibility. The demecolcine-induced concomitant alteration in p230 and intermediate filament organization raises also the interesting possibility that demecolcine may exert at least some of its effects on cellular functions by acting directly on p230.

We have previously shown (29) that the WGA-reactive material of the cytoskeletal preparations of cultured fibroblasts corresponds to the so-called surface lamina which includes detergent-resistant cell surface proteins, gangliosides, and peripheral plasma membrane components (5, 6, 39). In intact, paraformaldehyde-fixed cells the WGA-staining appears homogeneous while after prior detergent-treatment a gradual increase in the porosity of the staining pattern can be seen (29). This is probably due to passive lateral movement of the WGA-reactive cell surface components induced by detergent-extraction of most of the plasma membrane lipids. This compares well with what is seen in this study with anti-p230 after various fixations; after methanol, a rather homogeneous submembranous staining, and, after detergent, a more reticular staining can be seen. It seems that the former staining pattern of p230 is closer to the native situation. The latter, on the other hand, probably represents redistribution of p230 after the lipid depletion. This concomitant alteration in cell surface and subplasmalemmal organization suggests that there is a physical association between these components. In line with this, we have recently found that the WGA-receptor-p230-association is preserved also in cytochalasin B-treated, enucleated human fibroblasts (Laurila, P., I. Virtanen, and V.-P. Lehto, in preparation).

On the basis of the above results we suggest that p230 forms

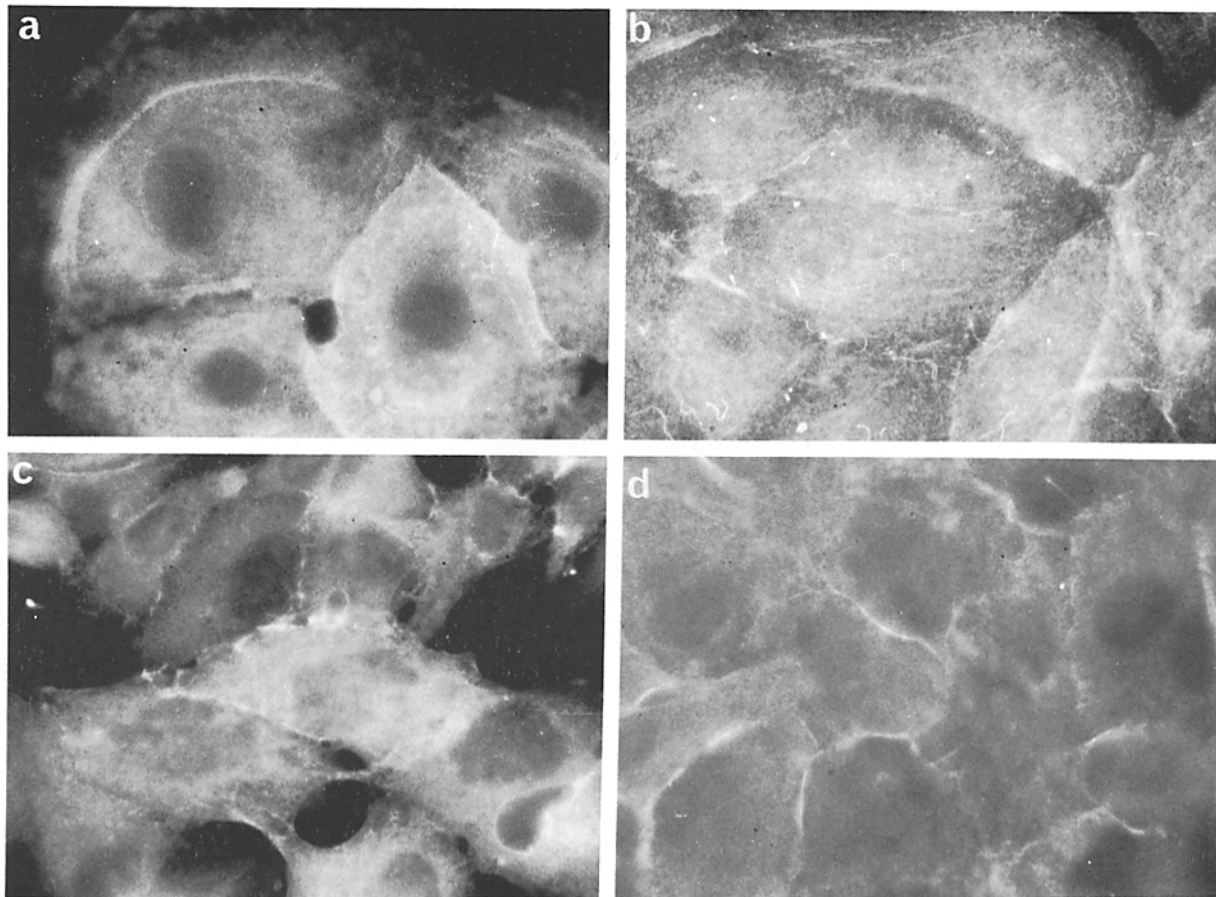


FIGURE 12 Fluorescence micrographs of human amnion epithelial cells (a), PTK<sub>2</sub>-cells (b), MDCK-cells (c), and bovine lens cells after staining with anti-p230. PF-NP-fixation.

a peripheral, subplasmalemmal layer which is part of the cell surface lamina. As such, p230 probably has direct interactions with plasma membrane—either hydrophobic association with plasma membrane lipids or protein-protein interactions with integral plasma membrane proteins—and also with cytoskeleton, especially with intermediate filaments.

Since the submission of this paper there have been reports on a nonerythroid protein of  $M_r$  over 200,000 daltons which has been shown to be immunologically cross-reactive and biochemically analogous to  $\alpha$ -spectrin of erythroid cells (45–47). On the basis of these results, we have shown in this study that also anti-p230 reacts with an adult human red blood cell polypeptide showing an electrophoretic mobility of  $\alpha$ -spectrin. Although we have not yet performed studies to detect possible

biochemical identity, it seems evident that p230 belongs to a family of spectrinlike molecules that currently includes brain fodrin (48), intestinal brush border proteins TW 260/240 (45), and brain spectrin (46). It is interesting that Glenney et al. (49) have recently shown that porcine brain fodrin, a spectrin-related protein, has actin-binding and cross-linking properties, pointing to a possible spectrinlike function in the submembranous cytoskeleton organization. Our results showing unaltered p230 in cytochalasin-B-treated and in monensin-exposed cells would suggest that, as an actin-binding protein, p230 would rather provide a mechanical support for microfilament formation than to be itself dependent on actin organization. Clearly, p230, together with other spectrin-related proteins, deserves further study as an important structural component

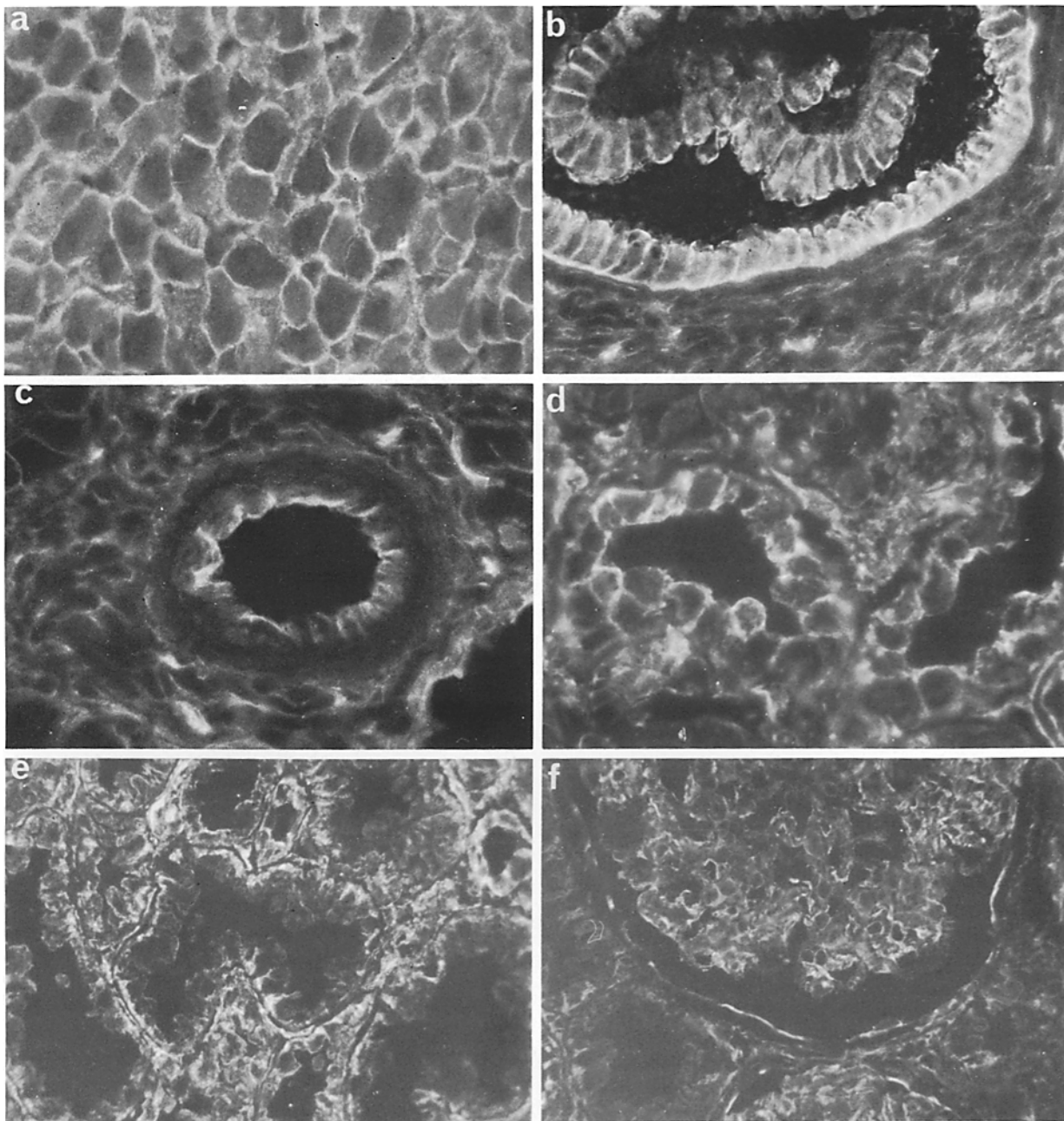


FIGURE 13 Fluorescence micrographs of human liver (a–c) and kidney (d–f) after staining with anti-p230. Methanol-fixation of cryostat sections.

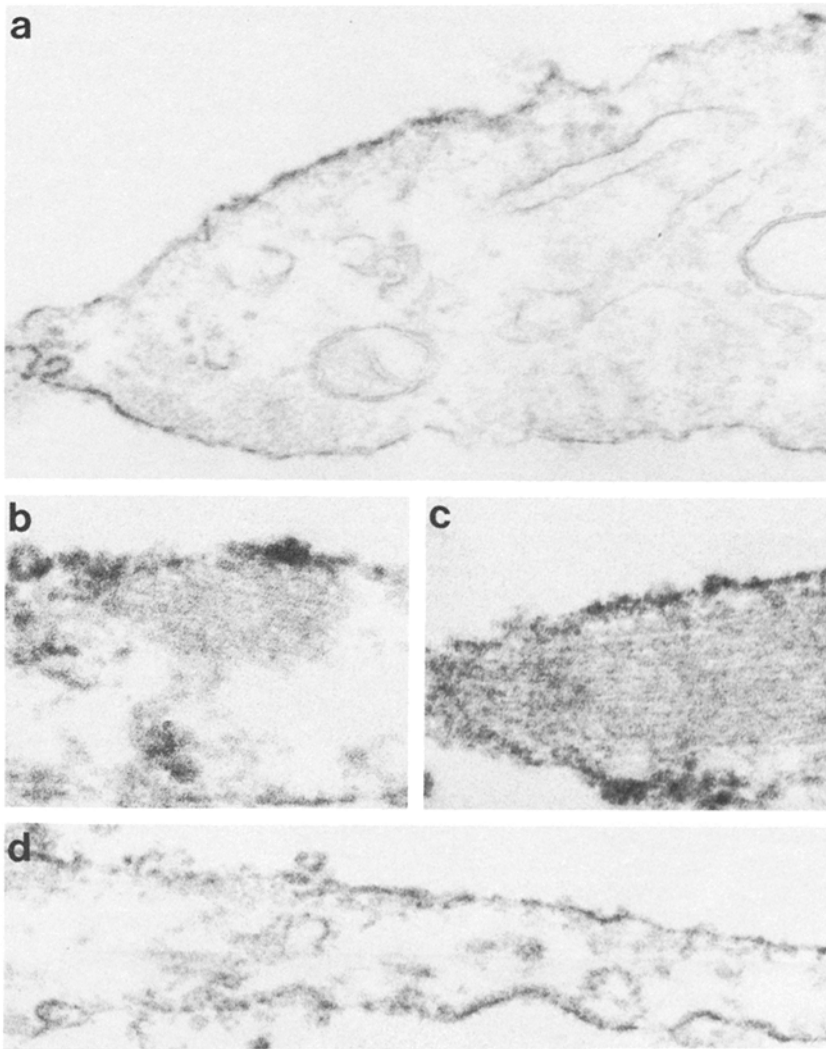


FIGURE 14 Immunoelectron micrographs of cultured fibroblasts after staining with anti-p230. Glutaraldehyde/0.01% Triton X-100-treated cells were stained with anti-p230 and then by using Vectastain as described in Materials and Methods.

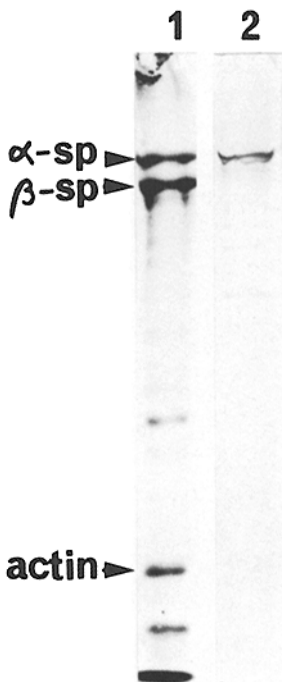


FIGURE 15 Identification of p230-cross-reactive material in human red blood cells (HRBC). HRBC-ghosts, further extracted with 0.5% Triton X-100, were electrophoresed, the polypeptides were transferred to nitrocellulose and then stained for proteins or exposed to anti-p230 as in Fig. 2. Lane 1 shows protein staining of the separated polypeptides and lane 2 the anti-p230-reactive polypeptides.

which may play, analogous to spectrin-complex in erythrocytes, a crucial role in the stabilization of cytoskeleton-plasma membrane associations in cultured adherent cells.

The skillful technical assistance of Ms. Pipsa Kaipainen, Ms. Raili Taavela and Ms. Ulla Waris is kindly acknowledged.

This study was supported by the Finnish Medical Research Council, the Sigrid Jusélius Foundation, the Finnish Cancer Research Fund, and the Association of the Finnish Life Insurance Companies.

Received for publication 24 May 1982, and in revised form 3 November 1982.

#### REFERENCES

1. Brown, S. W., Levinson, and J. A. Spudich. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. *J. Supramol. Struct.* 5:119-130.
2. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.)* 283:249-256.
3. Webster, R. E., D. Henderson, M. Osborn, and K. Weber. 1978. Three-dimensional electron microscopical visualization of the cytoskeleton of animal cells: immunoferritin identification of actin- and tubulin-containing structures. *Proc. Natl. Acad. Sci. U. S. A.* 75:5511-5515.
4. Osborn, M., and K. Weber. 1977. The detergent-resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. *Exp. Cell Res.* 106:339-349.
5. Ben-Ze'ev, A., A. Duerr, F. Solomon, and S. Penman. 1979. The outer boundary of the cytoskeleton; a lamina derived from the plasma membrane proteins. *Cell* 17:859-865.
6. Mescher, M. F., M. J. L. Jose, and S. P. Balk. 1981. Actin-containing matrix associated with plasma membrane of murine tumour and lymphoid cells. *Nature (Lond.)* 289:139-144.



7. Bretscher, A., and K. Weber. 1981. Fimbrin, a new microfilament-associated protein present in microvilli and other cell surface structures. *J. Cell Biol.* 86:335-340.
8. Bretscher, A., and K. Weber. 1979. Villin: the major microfilament-associated protein of the intestinal microvillus. *Proc. Natl. Acad. Sci. U. S. A.* 76:2321-2325.
9. Geiger, B. 1979. A 130 K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell.* 18:193-205.
10. Lazarides, E., and K. Burridge. 1975.  $\alpha$ -actinin: immunofluorescent localization of a muscle structural protein in non-muscle cells. *Cell.* 6:289-298.
11. Granger, B. L., and E. Lazarides. 1980. Synemin: a new high molecular weight protein associated with desmin and vimentin filaments in muscle. *Cell.* 22:727-738.
12. Breckler, J., and E. Lazarides. 1982. Isolation of a new high molecular weight protein associated with desmin and vimentin filaments from avian embryonic skeletal muscle. *J. Cell Biol.* 92:795-806.
13. Kim, H. L., L. J. Binnder, and J. L. Rosenbaum. 1979. The periodic association of MAP<sub>2</sub> with brain microtubules in vitro. *J. Cell Biol.* 80:266-276.
14. Pahlman, S., L. Odelstad, E. Larsson, G. Grotte, and K. Nilsson. 1981. Phenotypic changes of human neuroblastoma cells in culture induced by 12-O-tetradecanoyl-phorbol-13-acetate. *Int. J. Cancer.* 28:583-589.
15. Hormia, M., E. Linder, V.-P. Lehto, T. Vartio, R. A. Badley, and I. Virtanen. 1981. Vimentin filaments in cultured endothelial cells form butyrate-sensitive juxtanuclear masses after repeated subculture. *Exp. Cell Res.* 138:159-166.
16. Hovi, T., D. Mosher, and A. Vaheri. 1977. Cultured human monocytes synthesize and secrete 2-macroglobulin. *J. Exp. Med.* 145:1580-1592.
17. Lehto, V.-P., T. Hovi, T. Vartio, R. A. Badley, and I. Virtanen. 1982. Reorganization of cytoskeletal and contractile elements during transition of human monocytes into adherent macrophages. *Lab. Invest.* 47:391-399.
18. Virtanen, I., V.-P. Lehto, E. Lehtonen, T. Vartio, S. Stenman, P. Kurki, O. Wager, J. V. Small, D. Dahl, and R. A. Badley. 1981. Expression of intermediate filaments in cultured cells. *J. Cell Sci.* 50:45-63.
19. Steck, T. L., R. S. Weinstein, J. H. Straus, and D. F. H. Wallach. 1970. Inside-out red cell membrane vesicles: preparation and purification. *Science (Wash. DC).* 168:255-257.
20. Lehto, V.-P., I. Virtanen, and P. Kurki. 1978. Intermediate filaments anchor the nuclei in nuclear monolayers of cultured human fibroblasts. *Nature (Lond.)* 272:175-177.
21. Virtanen, I., T. Vartio, R. A. Badley, and V.-P. Lehto. 1982. Fibronectin in adhesion, spreading and cytoskeletal organization of cultured fibroblasts. *Nature (Lond.)* 298:660-663.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
23. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of <sup>3</sup>H and <sup>14</sup>C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335-341.
24. Towbin, H., T. Staehelin, and L. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U. S. A.* 76:4350-4354.
25. Laurila, P., I. Virtanen, J. Wartiovaara, and S. Stenman. 1978. Fluorescent antibodies and lectins stain intracellular structures in fixed cells treated with nonionic detergent. *J. Histochem. Cytochem.* 26:251-256.
26. Feramisco, J. R., and K. Burridge. 1980. A rapid purification of  $\alpha$ -actinin, fimbrin and a 130,000-dalton protein from smooth muscle. *J. Biol. Chem.* 255:1194-1199.
27. Badley, R. A., A. Woods, L. Carruthers, and D. A. Rees. 1978. Cytoskeleton changes in fibroblast adhesion and detachment. *J. Cell Sci.* 43:379-380.
28. Barak, L. S., R. R. Yocum, E. A. Nothnagel, and W. W. Webb. 1980. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole phalloidin. *Proc. Natl. Acad. Sci. U. S. A.* 77:980-984.
29. Lehto, V.-P., T. Vartio, R. A. Badley, and I. Virtanen. 1982. Characterization of a detergent-resistant surface lamina in cultured human fibroblasts. *Exp. Cell Res.* In press.
30. Ramaekers, F. C. S. and R. Bloemendal. 1982. Cytoskeletal and contractile structures in lens cell differentiation. *In Molecular and Cellular Biology of the Eye Lens.* H. Bloemendal, editor. John Wiley & Sons, New York. 86-135.
31. Geisler, N., and K. Weber. 1981. Isolation of polymerization-competent vimentin from porcine eye lens tissue. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 125:253-256.
32. Virtanen, I., V.-P. Lehto, E. Lehtonen, and R. A. Badley. 1980. Organization of intermediate filaments in cultured fibroblasts upon distribution of microtubules by cold treatment. *Eur. J. Cell Biol.* 23:80-84.
33. Blöse, S. H., M. L. Shelanski, and S. Chacko. 1977. Localization of bovine brain filament antibody on intermediate (100 Å) filaments in guinea pig vascular endothelial cells and in chick cardiac muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:662-667.
34. Marchesi, V. T. 1979. Spectrin: present status of a putative cytoskeletal protein of the red cell membrane. *J. Membr. Biol.* 51:101-131.
35. Branton, D., C. M. Cohen, and J. Tyler. 1981. Interaction of cytoskeletal proteins on the human erythrocyte membrane. *Cell.* 24:24-32.
36. Helenius, A., D. R. MiCaslin, E. Fries, and C. Tanford. 1979. Properties of detergents. *Methods Enzymol.* 55:734-749.
37. Shterline, P., and C. R. Hopkins. 1981. Transmembrane linkage between surface glycoproteins and components of the cytoplasm in neutrophil leukocytes. *J. Cell Biol.* 90:743-754.
38. Pober, J. S., B. C. Guild, J. L. Strominger, and W. R. Veatch. 1981. Purification of HLA-A2 antigen, fluorescent labeling of its intracellular region, and demonstration of an interaction between fluorescently labeled HLA-A2 antigen in lymphoblastoid cell cytoskeleton proteins in vitro. *Biochemistry* 20:5625-5633.
39. Sreuli, C. H., B. Patel, and D. R. Critchley. 1981. The cholera toxin receptor ganglioside GM1 remains associated with Triton X-100 cytoskeletons of BALB/c-3T3 cells. *Exp. Cell Res.* 136:247-254.
40. Painter, R. G., M. Ginsberg, and B. Jaques. 1982. Concanavalin A induces interactions between surface glycoproteins and the platelet cytoskeleton. *J. Cell Biol.* 92:565-573.
41. Singer, I. I. 1982. Association of fibronectin and vinculin with focal contacts and stress fiber in stationary hamster fibroblasts. *J. Cell Biol.* 92:398-408.
42. Lehto, V.-P. 1982. 140 000 dalton surface glycoprotein. A plasma membrane component of the detergent-resistant cytoskeletal preparations of cultured human fibroblasts. *Exp. Cell Res.* In press.
43. Ramaekers, F. C. S., I. Dunia, H. J. Dodemont, E. L. Benedetti, and H. Bloemendal. 1982. Lenticular intermediate-sized filaments: biosynthesis and interaction with plasma membrane. *Proc. Natl. Acad. Sci. U. S. A.* 79:3208-3212.
44. Granger, B. L., and E. Lazarides. 1982. Structural associations of synemin and vimentin filaments in avian erythrocytes revealed by immunoelectron microscopy. *Cell.* 30:263-275.
45. Glenney, Jr. J. R., P. Glenney, and K. Weber. 1982. Erythroid spectrin, brain fodrin, and intestinal brush border proteins (TW-260/240) are related molecules containing a common calmodulin-binding subunit bound to a variant cell type-specific subunit. *Proc. Natl. Acad. Sci. U. S. A.* 79:4002-4005.
46. Bennett, H. V., J. Davis, and W. E. Fowler. 1982. Brain spectrin, a membrane-associated protein related in structure and function to erythrocyte spectrin. *Nature (Lond.)* 299:126-131.
47. Repasky, E. A., B. L. Granger, and E. Lazarides. 1982. Widespread occurrence of avian spectrin in nonerythroid cells. *Cell.* 29:821-833.
48. Levine, J., and M. Willard. 1981. Fodrin: axonally transported polypeptides associated with the internal periphery of many cells. *J. Cell Biol.* 90:631-643.
49. Glenney, Jr. J. R., P. Glenney, and K. Weber. 1982. F-actin-binding and cross-linking properties of porcine brain fodrin, a spectrin-related molecule. *J. Biol. Chem.* 257:9781-9787.