Most Iodinatable Fibroblast Surface Proteins Accompany the Cytoplast Membrane during Cytochalasin B-mediated Enucleation of Chick Embryo Fibroblasts

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ABSTRACT Six different proteins are found to be reproducibly exposed on the cell surface of chicken embryo fibroblasts (CEF) by the criterion of lactoperoxidase-catalyzed iodination (250,000, 185,000, 130,000, 100,000, 87,000, and 75,000 daltons). We wondered whether cell enucleation might lead to a differential partition of these surface proteins with the karyoplast or cytoplast membrane.

We found that there is a marked enrichment of most iodinatable cell surface proteins in the cytoplast after cytochalasin-mediated enucleation of cell monolayers. Nearly all the iodinatable fibronectin remains with the cytoplast. Of the six labeled proteins, the karyoplast membrane contains a small amount of the 130 kdalton protein as well as trace levels of the 100-, 85-, and 75-kdalton proteins. Proteolysis or selective shedding of membrane proteins were not significant factors in the relative exclusion of iodinatable membrane proteins from the karyoplast.

The cytoplast could replace some exposed membrane proteins after removal by trypsinization; however, fibronectin was not detectable within 10 h. That the karyoplast was not capable of membrane protein synthesis and/or insertion was suggested by the lack of any change in the labeling pattern of karyoplasts up to 8-h incubation after enucleation.

A variety of control studies indicated that the surface proteins identified in this report were cell-derived and not adsorbed serum components. That some of the iodinatable proteins are intrinsic membrane proteins was suggested by their resistance to removal by conditions thought to extract extrinsic membrane proteins (i.e., low salt, high salt, and NaOH washes).

Lack of effect of cytoskeletal disrupting agents (preliminary evidence) suggests the nonrandom partition of membrane proteins may depend on anchoring of membrane proteins by a system(s) in the cytoplast other than intact microtubules and microfilaments.

Fibroblasts of several types grown in monolayer culture, treated with cytochalasin B and subjected to centrifugal force are separated into two parts, the cytoplast and the karyoplast (31). The cytoplast, which remains adherent to the substratum, contains all the organelles of the intact cell with the exception of the nucleus. The karyoplast contains the nucleus, about 10–30% of the cytoplasm with most organelles in diminished amounts (28), and a limiting membrane derived from the plasma membrane of the parent cell (26). The mechanism of this nuclear expulsion appears to begin with an outpocketing of the plasma membrane containing the nucleus (26). As centrifugation proceeds, the stalk formed by this process ultimately breaks to produce two entities.

Studies have indicated that cytoplasts, after recovery from cytochalasin B, behave in a similar fashion to normal cells. They exhibit motility, contact inhibition, and normal morphology (7), as well as continuing to synthesize proteins (25). The freed karyoplasts are spherical and do not reattach to a substrate although isolated exceptions have been reported (16, 26).

By morphometric analysis of suspended karyoplast and cytoplasts, we determined that the apparent surface area of the average karyoplast from chicken embryo fibroblasts is somewhat less than the surface area of the average cytoplast (250 μm^2 vs. 450 μm^2). If a membrane-associated protein were independent of other membrane proteins and of any "anchorage factors," it might be expected to exhibit a random distribution with ~35% of the surface protein partitioning with

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 SEPTEMBER 1982 557-564

the karyoplast and ~65% with the cytoplast. Conversely, if a membrane protein were constrained by an interaction with a system which remained with the cytoplast an asymmetric distribution between the cytoplast and karyoplast would result.

The partition of tightly associated surface proteins between karyoplast and cytoplast plasma membrane was investigated using lactoperoxidase catalyzed iodination to label and follow those proteins.

MATERIALS AND METHODS

Cells

Primary chick embryo fibroblast (CEF) cultures were obtained by trypsinization (0.25%, wt/vol) of eviscerated 10-d chick embryos. The cells were grown in H-21 medium (Gibco, Grand Island Biological Co., Grand Island, NY) supplemented with 5% calf serum, 50 U/ml penicillin, and 50 μg/ml streptomycin on 100-mm plastic petri dishes at 37°C in an atmosphere of 5% CO_2. Subcultures obtained from these primaries by trypsinization (0.05%, wt/vol) were designated first passage and were subsequently subcultured every 4–5 d. Cells used for the studies described here were generally from second to fourth passage.

Surface Labeling

Fibroblasts were enzymatically iodinated by the coupled glucose oxidase-lactoperoxidase (LPO) method (9, 19) after removing the medium and washing the cell monolayer three times with phosphate buffered saline (PBS; 50 mM phosphate pH 7.4, 125 mM NaCl) containing 1 mM EDTA (PBS-EDTA). 2 ml of PBS containing 2 U of lactoperoxidase (Sigma Chemical Co., St. Louis, MO) and 1 U of glucose oxidase (Type A, Calbiochem-Behring Corp., La Jolla, CA) was then added to the cultures followed by 50 μl of PBS containing 12.5 μmol glucose and 12.5 nmol KI. Carrier iodide was found to be essential for maximal iodination (22) when adding <500 μCi/ml ^{125}I (based on a ^{125}I specific activity of ~17 Ci/mg). After incubation at room temperature for 10 min, the reaction mixture was removed, and the monolayer washed first with PBS containing 10 mM KI and then five times with PBS. Longer incubation times were avoided to minimize internalization of the glucose oxidase and lactoperoxidase.

Calf serum was labeled to high specific activity with ^{125}I by the chloramine-T procedure (8) and, after removal of free isotope by gel filtration on Sephadex G-25, was analyzed by 2-D gel electrophoresis and autoradiography. In addition, intact fibroblasts were incubated with this labeled serum and washed with PBS to determine the extent to which serum proteins adsorbed to the cells.

Fibroblasts were metabolically labeled with [^{35}S]methionine by incubation for 18 h in medium containing 25 μCi/ml of this isotope and 20% of the normal concentration of methionine. Cells labeled in this fashion were then analyzed by 2-D gel electrophoresis and autoradiography.

Enucleation

Enucleation was carried out 24–36 h after fibroblasts were seeded into polylysine-treated (17) 25 cm^2 flasks (Corning Glassworks, Corning, NY) at a density of 4–6 × 10^4 cells/cm^2. The flasks of near confluent cells were completely filled (~75 ml) with H-21 medium containing 1% calf serum, 1% DMSO and 10 μg/ml cytochalasin B (Aldrich Chemical Co., Milwaukee, WI). To inhibit possible proteolysis, 2 mM phenylmethyl sulfonylfluoride (Sigma Chemical Co.) was included in some studies but this addition produced no qualitative differences in electrophoretic gel patterns although 10–20% less radiolabel was released into the medium from both unenucleated and enucleated cells. After a 15-min incubation at 37°C, the cells were subjected to centrifugation at 10,000 *g* for 1 h in a GSA rotor (Sorvall RC-2 centrifuge; DuPont Instruments, Newtown, CT) at 37°C, using a water cushion for the flasks. This resulted in enucleation of 90–95% of the cells as scored by light microscopy. The cytochalasin B-containing medium was removed and the karyoplasts were collected. These were washed with PBS and either lysed in SDS-containing buffer immediately or washed and incubated in fresh medium. If the cells had been iodinated before enucleation, the cytoplasts were washed with PBS and solubilized directly in SDS buffer. However, if labeling was to take place after enucleation, they were first incubated in fresh medium for a minimum of 30 min to permit morphologic recovery from the cytochalasin B.

Morphometric Size Analysis of Karyoplasts and Cytoplasts

The relative sizes of karyoplasts and suspended cytoplasts were determined by comparison to particles of known size. After enucleation, karyoplasts were collected and cytoplasts were brought into suspension by brief trypsinization (0.05% trypsin for 5 min). Wet mounts were made of these suspensions and they were photographed at ×320. Suspensions were also made of latex beads (6.8 μm, 13.5 μm, and 20 μm diameter, Particle Information Service, Los Altos, CA) and photographed under identical conditions. Diameters of the karyoplasts and cytoplasts were then determined by interpolation from the standard plot. The surface areas of these spherical entities were calculated using the surface area equation for a sphere.

Low Salt, High Salt, and Sodium Hydroxide Extraction of Intact Cells and Membranes

Whole cells and membranes prepared by the procedure of Brunette and Till (4) were extracted under conditions of low salt, high salt, or 0.007 M NaOH to remove soluble proteins and nonintrinsic membrane proteins (12, 28). After iodination, the cells were washed three times with PBS containing 10 mM KI and twice with PBS. The monolayers were then treated with two brief rinses (6 ml each) followed by a slower rinse (3 ml for 3–5 min) at 4°C with one of the following: (*a*) complete Dulbecco's minimum essential medium, (*b*) 0.5 M NaCl, (*c*) 0.008 M NaCl, 1 mM phosphate, pH 7.5, or (*d*) 0.007 M NaOH, pH 11.5. The residual material from these extractions was then prepared for 1-D SDS electrophoresis by the addition of 0.4 ml of SDS lysis buffer.

This protocol was also carried out on cells which had been scraped into the extraction buffer and with isolated membranes. Insoluble material was collected by centrifugation at 12,000 *g* for 45 min in distilled water and dissolved in SDS lysis buffer.

Preparation of Substratum Attached Material

Substratum attached material (SAM) (5) was prepared after lactoperoxidase iodination. Flasks containing labeled whole cells were filled with PBS containing 5 mM EGTA and centrifuged at 8,500 RPM in a similar fashion to a standard enucleation. This resulted in detachment of most of the cells from the substratum and subsequent microscopic examination indicated that the material left represented cellular attachment sites or "footpads" (5). This material was solubilized in SDS lysis buffer and analyzed by SDS electrophoresis.

Electrophoresis of Labeled Samples

Samples were prepared for both one and two-dimensional gel electrophoresis by lysis in SDS containing buffer (2.3% SDS, 60 mM Tris-Cl pH 6.8, 5% mercaptoethanol, 2 mM EDTA). If samples were to be analyzed by 2-D electrophoresis, 2 vol of a solution comprising 9.5 M urea, 8% nonidet P-40 (NP-40) (Particle Data Laboratories Ltd., Elmhurst, IL), and 5% mercaptoethanol (2) was subsequently added. SDS-PAGE was carried out by the method of Laemmli (15). Two-dimensional gel electrophoresis was as described by O'Farrell (24) and modified by Ames and Nikaido (2). One-dimensional gels comprised a linear 7.5–15% gradient unless otherwise noted. The second dimension of 2-D electrophoresis was on 10% polyacrylamide gels.

Gels were stained with Coomassie Blue by the method of Fairbanks et al. (6), destained, and dried onto filter paper. The dried gels were then exposed to Cronex 4 x-ray film for 48 h.

RESULTS

Variables in the Lactoperoxidase Iodination of Intact Chick Embryo Fibroblasts

Intact chick embryo fibroblasts labeled by lactoperoxidase iodination were examined by one- and two-dimensional SDS PAGE. A highly reproducible pattern of ^{125}I-labeled membrane proteins included bands at 250,000, 185,000, 130,000, 100,000, 87,000, and 75,000 daltons (Fig. 1 *c* and *d*). Other membrane bands (generally smaller molecular weight) were not always reproducible and will not be considered in this paper. The labeling of the 250,000 dalton (250 kd) protein (fibronectin, LETS protein) was found to increase with increasing cell density as previously described (10). The labeling of 130 kd and 100 kd also increases with cell density. The bands at 87 kd and 75 kd decreased somewhat with increasing cell density (data not shown). The diagonal array of the 130 kd species on two-dimensional gel electrophoresis (Fig. 1 *d*) suggested that it might be a sialoglycoprotein with considerable microhetero-

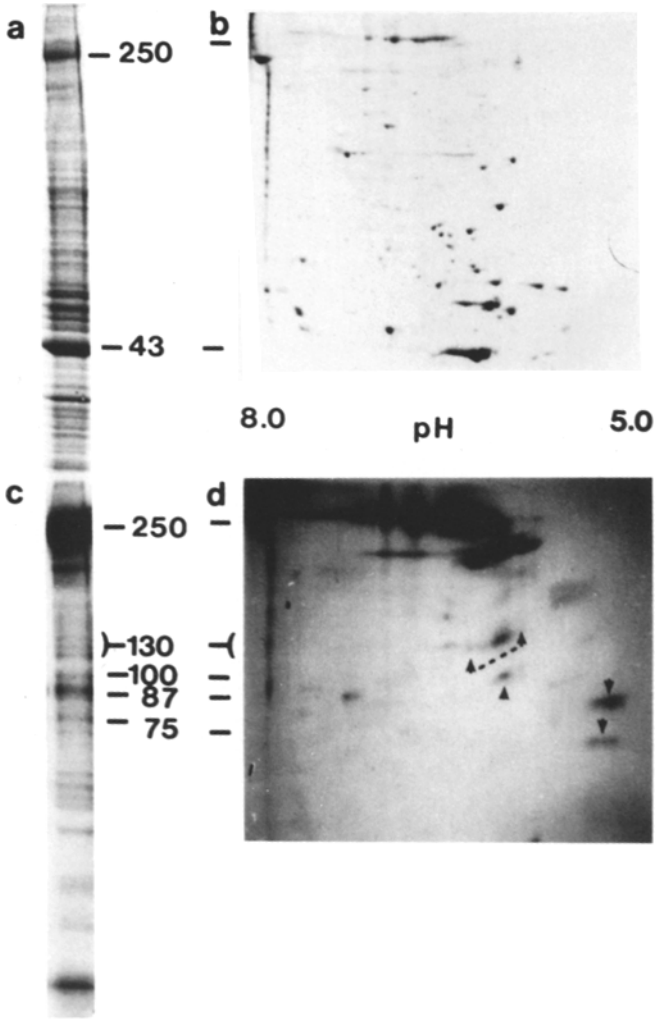


FIGURE 1 One and two-dimensional gel analysis of surface proteins of chick embryo fibroblasts. Cells were plated 24 h before labeling at a density of 4×10^4 cells/cm^2. Lactoperoxidase catalyzed iodination was carried out using 50 μCi of ^{125}I in the presence of 3.5 μM I^- as carrier. Cells were lysed in 200 μl SDS buffer/flask an 25 μl aliquots were analyzed by either discontinuous SDS PAGE (15) (*a* and *c*) or a combination of isoelectric focusing and SDS PAGE as described by O'Farrell (24) (*b* and *d*). The gels were stained for protein with Coomassie Blue and dried (*a* and *b*), then subjected to autoradiography (*c* and *d*). SDS-containing gels were all 10% polyacrylamide. Origins of the 2-D gels are in the upper left-hand corner.

geneity in its sialylation (3). This may explain why multiple bands were often seen at this apparent molecular weight on one-dimensional gels (Fig. 1*c*). For all of the enucleation experiments described, we plated the cells at about the same cell density represented here (10^6 cells/25 cm^2 flask 24–36 h before radiolabeling or enucleation).

It was important to establish that none of these labeled proteins represented secreted, extracellular proteins or adsorbed serum proteins not removed by the washing procedure. Whole serum was iodinated to high specific activity by the chloramine-T method and either analyzed directly by 2-D gel electrophoresis or incubated with intact chicken embryo fibroblasts before lysis and 2-D gel electrophoresis. These control studies indicated that only a small amount of labeled albumin remained bound to the cells after the washing procedure. No other serum proteins could be detected. In addition, direct analysis of iodinated serum indicated that the proteins were generally found in a more basic region of the 2-D gel (pI >7) than the labeled surface proteins considered in this study. These data suggested that the proteins under consideration are cellular and not derived from the serum. Further support for this concept was obtained by 2-D gel electrophoretic analysis of membranes prepared from cells by the method of Brunette and Till (4) which had been metabolically labeled with [^{35}S]-methionine. The putative surface proteins as identified by lactoperoxidase iodination were present in these preparations and had incorporated radioactive methionine. This confirms that these proteins are cellular in origin.

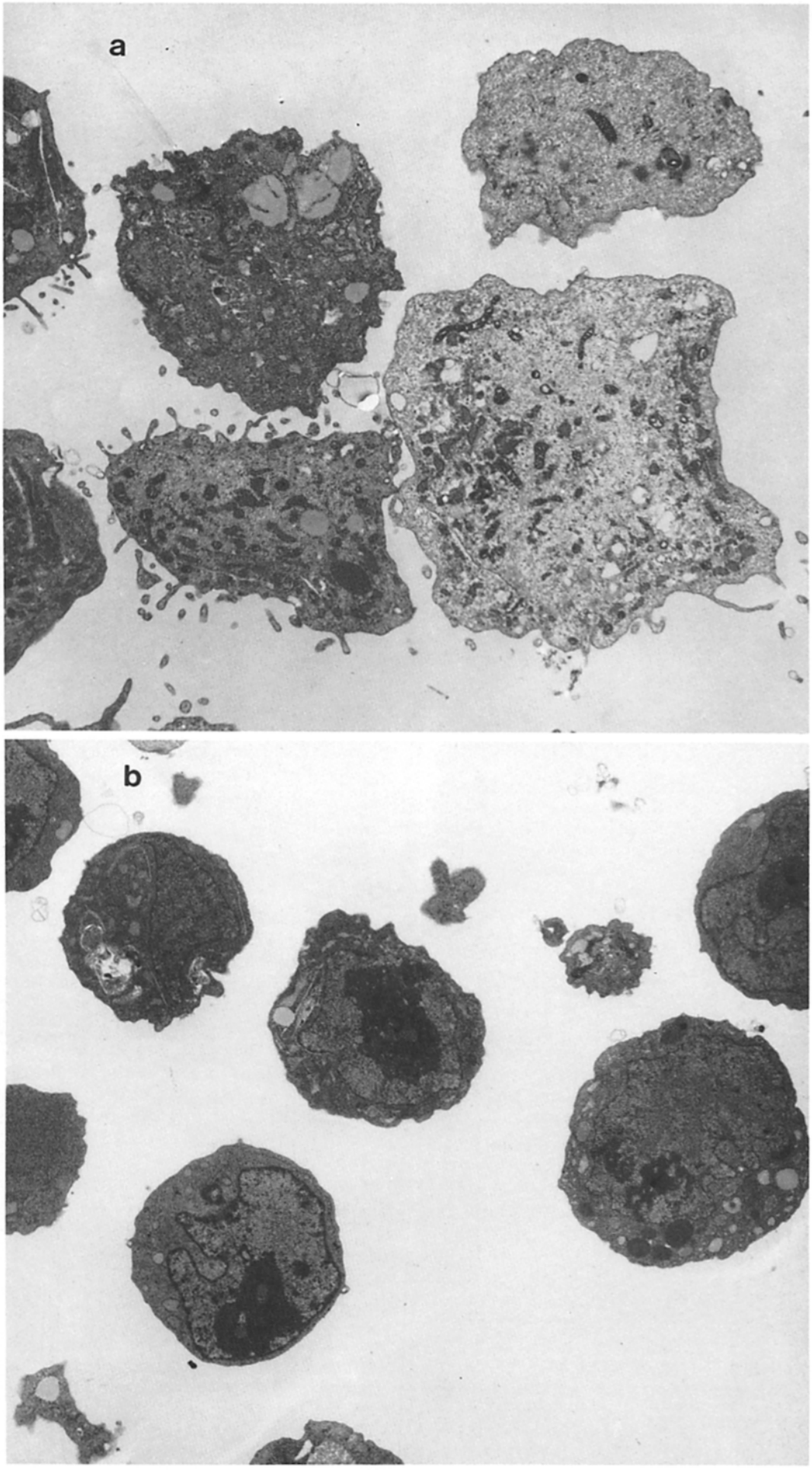
Proteins which are associated with these membrane preparations are not necessarily membrane proteins but may be bound cytoskeletal or cytoplasmic elements, so it was important to further confirm the membrane origin of the iodinated proteins. To investigate this, whole cells were extracted under hypotonic and hypertonic conditions as well as with 0.007 M NaOH (pH 11.5) which has been reported to remove extrinsic and/or adventitiously bound proteins from the plasma membrane (12, 28). Under none of the above conditions were these iodinated proteins extracted. Membranes prepared as above also retained these proteins under all conditions.

The possibility was also considered that the labeled 75-kd band might represent autoiodinated lactoperoxidase (LPO) bound to the membrane. To test this, lactoperoxidase and glucose oxidase were radioiodinated to high specific activity in a cell-free system using the chloramine-T reaction (8). After gel filtration (Sephadex G-25) to remove free ^{125}I, the iodinated enzymes were incubated with fibroblasts and washed under conditions identical to the standard LPO labeling system. Analysis by 2-D gel electrophoresis indicated that autoiodinated LPO focused in a much more basic region of the gel ($\approx$pI = 7.5) than the proteins considered here (Fig. 1*d*) and that only trace amounts of the enzyme were bound to the cell. We conclude that the proteins iodinated by lactoperoxidase in intact cells are cell-derived and membrane in origin.

Enucleation of Lactoperoxidase Iodinated Chick Embryo Fibroblasts and the Distribution of Surface Proteins

The distribution of the iodinated surface proteins between cytoplast and karyoplast after enucleation was examined. CEF at cell densities of 6–8 $\times$ 10^4 cells/cm^2 were iodinated by lactoperoxidase, incubated with cytochalasin B for 15 min and then enucleated by centrifugation at 10,000 *g* for 1 h. Both light and electron microscopy of the cytoplast monolayer revealed that enucleation efficiency was between 90–95%. The unattached karyoplasts were seen by transmission electron microscopy to be membrane bound with reduced cytoplasmic content relative to the cytoplast (Fig. 2).

SDS gel electrophoresis carried out on the cytoplast and karyoplast preparations showed that, although the cytoplasts that remained adherent to the substratum exhibited all of the labeled surface proteins of the whole cell (Fig. 3*b*), few labeled bands could be seen in the karyoplasts (Fig. 3*d*). This was consistent with the observation that <5% of the ^{125}I activity associated with the cytoplasts was found in the karyoplast fraction. The major detectable surface protein in the karyoplast is present as a faint band at 130–135 kd but this is greatly reduced relative to the amount present in the cytoplast membrane. This relative lack of iodinated protein is seen even when about four to five times as many karyoplasts as cytoplasts were represented in the gel. The average cytoplasmic content of


a
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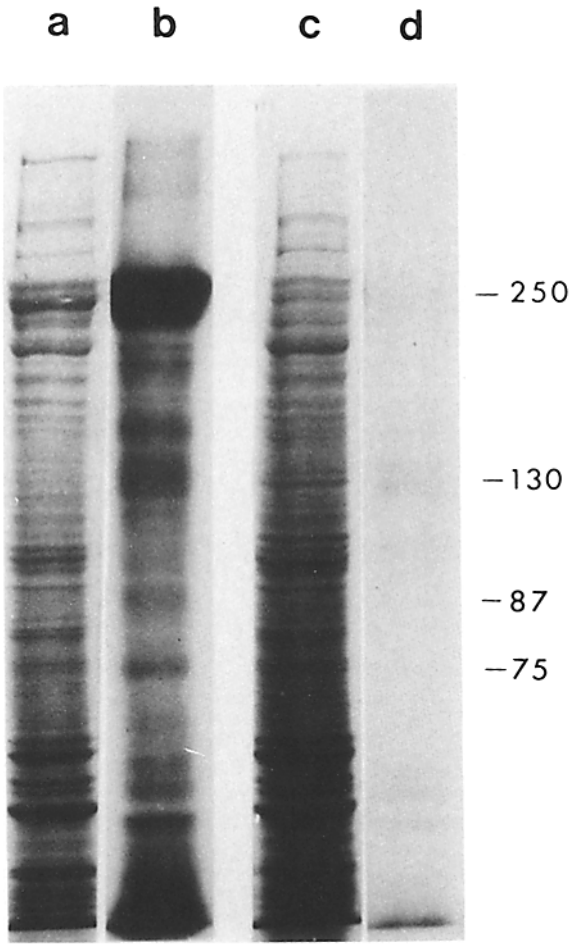

FIGURE 3 Distribution of surface label after enucleation. Chick embryo fibroblasts at a density of ~6 × 10^4 cells/cm^2 were iodinated as described using 30 μCi of ^{125}I for a 25 cm^2 flask. After enucleation, the attached cytoplasts (*a* and *b*) were washed extensively with cold PBS and solubilized in 100 μl/flask of SDS containing buffer. The free karyoplasts from six flasks (*c* and *d*) were collected by centrifugation, washed in PBS, and lysed in 150 μl of SDS buffer. 15-μl aliquots of these samples (representing 3 × 10^5 cytoplasts and 12 × 10^5 karyoplasts) were then analyzed by SDS PAGE on gradient (7.5–15%) gels. The gels were stained with Coomassie Blue, dried (*a* and *c*) and then subjected to autoradiography for 48 h (*b* and *d*).

karyoplasts has been estimated to be ~10% of that of the cytoplasts (33) although electron microscopy of our preparations showed variation with cytoplasmic content ranging between 10–30% (Fig. 2). Morphometric analysis of the suspended cytoplasts and karyoplasts indicated that the average karyoplast diameter was 8.5 μm (range from 7–9 μm) while cytoplasts averaged 12 μm in diameter with somewhat more variability (range from 8–18 μm) consistent with the variability in the size of the intact cell. The surface area of these roughly spherical entities was then calculated to be 225 μm^2 (karyoplast) and 450 μm^2 (cytoplast). This indicated that ~35% of the original cell surface area was associated with the karyoplast while 65% remained with the cytoplast.

Labeled fibroblasts treated with cytochalasin B but not centrifuged (i.e., <1% enucleation [25]) showed labeling patterns similar to untreated cells suggesting that cytochalasin alone was incapable of releasing or otherwise altering labeled surface proteins. The addition of cholchicine (4 μg/ml) or vinblastine (5 μg/ml) (25) along with cytochalasin B had no effect on the partition of the surface proteins (data not shown).

We considered whether the relative lack of LPO-labeled surface proteins on the karyoplast could result from a loss through proteolysis by running duplicate enucleations in the presence and absence of 2 mM phenylmethylsulfonyl fluoride (PMSF). The inclusion of PMSF before and during enucleation resulted in a small increase (10–20%) in the total recovery of radioactivity associated with the cytoplast preparations; however, no additional recovery was noted in the radioactivity associated with the karyoplast preparations. The SDS gel patterns of the labeled proteins in the presence of PMSF were indistinguishable from the patterns obtained in its absence (data not shown).

Aliquots of the enucleation media after centrifugation were fractionated on an AcA 34 (LKB Instruments, Uppsala, Sweden) column to determine if significant quantities of fibronectin or other proteins were being shed during cytochalasin B incubation and/or centrifugation. Most of the radiolabel found in the media was eluted in the total bed volume indicating a molecular weight below 20 kd. A small amount of label (~2%) was observed to elute with an apparent molecular weight of 200–250 kd. This was most likely labeled fibronectin which had been released as a result of the cytochalasin B treatment (1, 14); the release of labeled fibronectin from enucleated cell preparations was identical to that released from unenucleated cells treated with an equivalent amount of cytochalasin B but not centrifuged. In both cases, <10% of the total labeled fibronectin was found in the medium. As this total amount of released fibronectin was insufficient to account for the absence of fibronectin from the karyoplast membrane, a "shedding" mechanism from the karyoplast was ruled out.

Analysis of Substratum-associated Material

It was important to determine whether these cell surface proteins were specifically associated with the substratum-associated material (SAM) (5) or the adhesion plaques of the cells. Such an association would prevent partitioning of the proteins into the karyoplast membrane and could account for the observed results. It has been noted that fibronectin is involved in the SAM complex although this pool may be less susceptible to LPO iodination (23). Labeled intact cells were treated with 5 mM EGTA in PBS and subjected to centrifugation. This resulted in detachment of most of the cells from the substratum leaving behind fibrillar material and small blebs of cytoplasm that were considered to represent SAM. SDS PAGE analysis of this material indicated that although some labeled fibronectin remained associated with the SAM, the great majority was found in the detached cells (Fig. 4). The other labeled surface proteins were undetectable in the SAM fraction. From this we conclude that the surface proteins identified by lactoperoxidase labeling are not exclusively involved in cell adhesion and thus that direct association of these proteins with the substratum cannot account for the asymmetric distribution that is observed.

FIGURE 2 Electron microscopy of cytoplasts and karyoplasts. After enucleation, cytoplasts (*a*) were harvested by trypsinization (0.25%) and scraping and along with karyoplasts (*b*) were fixed in 3% glutaraldehyde in 0.2 M phosphate buffer and postfixed in osmium tetraoxide. The pellets were dehydrated and embedded in Epon mixture. Sections were counterstained with uranyl acetate and lead citrate and observed and photographed in a Philips 201 electron microscope. × 3,500.

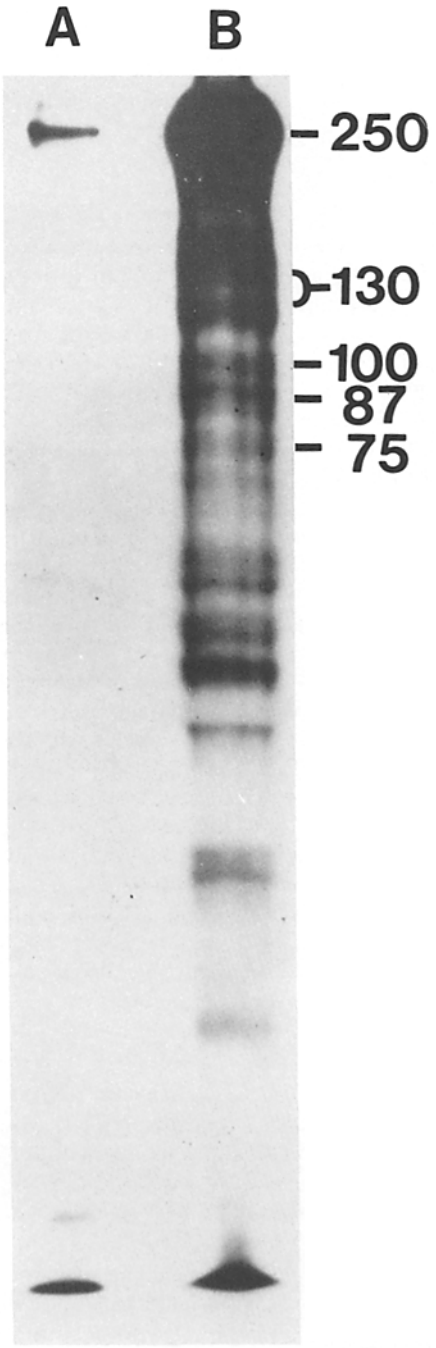

FIGURE 4 Analysis of substratum-associated material. After lactoperoxidase iodination the monolayer was washed with PBS and the flasks were filled with PBS containing 5 mM EGTA. The flasks were then centrifuged in a similar fashion to a standard enucleation. This resulted in detachment of most of the cells from the substratum. The material remaining attached to the substratum (SAM) was solubilized in 200 μl of SDS lysis buffer and 30 μl was analyzed by SDS PAGE (*a*). This can be compared to the pattern seen with whole cells (*b*) when analyzed by the same procedures. Radiolabel migrating with the front may be labeled lipid (18).

Lactoperoxidase Iodination of Cytoplasts and Karyoplasts after Enucleation

Unlabeled chick fibroblasts were subjected to cytochalasin B-induced enucleation and the cytoplasts subsequently labeled by lactoperoxidase iodination (Fig. 5*a*). Suspended karyoplasts which were surface-labeled using 10 fold higher concentrations

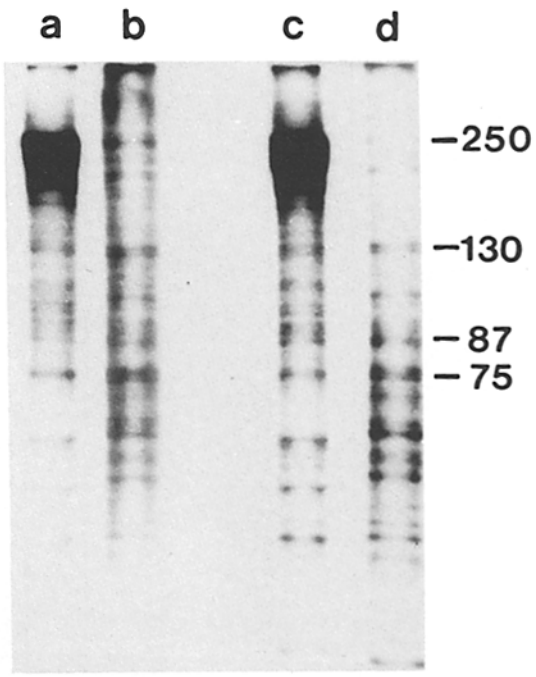

FIGURE 5 Surface labeling of cytoplasts and karyoplasts after enucleation. Fibroblasts were enucleated as described and the cytoplasts (*a* and *c*) and karyoplasts (*b* and *d*) were either labeled immediately by lactoperoxidase iodination (*a* and *b*) or were incubated in fresh medium (without cytochalasin B) for 8 h before labeling (*c* and *d*). In this experiment, the amount of ^{125}I used to label the karyoplast preparations was 250 μCi while 25 μCi was used to label the cytoplasts. Other than this the conditions were identical to the previous experiments. Autoradiography of the dried gels was for 48 h. Only the autoradiograms are shown.

of ^{125}I than for an equivalent number of cytoplasts did demonstrate detectable labeling of certain of the surface proteins (Fig. 5*b*). These included the 130-, 100-, 87-, and 75-kd proteins; however, very little labeling of fibronectin could be detected (Fig. 5*b*).

Studies were then carried out to determine if the net reappearance or loss of surface proteins could occur with time in either cytoplasts or karyoplasts. After enucleation, the unlabeled cytoplast layers were washed and incubated in fresh medium for 8 h. Karyoplasts were collected, washed, and incubated as a suspension in fresh medium. Surface proteins were then labeled by lactoperoxidase catalyzed iodination. As indicated, no apparent changes occur over 8 h (Fig. 5*c* and *d*). Specifically, fibronectin does not appear on the surface of the karyoplast and there is no net loss of this or other surface proteins from the cytoplast over the course of the experiment.

Reappearance of Surface Proteins in Cytoplasts after Trypsinization

The preceding data indicate that, within 8 h after enucleation, there is no net loss of iodinatable proteins from the cytoplast. However, the experiment was not capable of distinguishing between preexisting and newly synthesized surface proteins. In other words, did the level of iodinatable fibronectin and other proteins in the cytoplast reflect constant steady state levels, or simply stability during the time course of the experiment?

To investigate the appearance of surface proteins, the cytoplasts were removed from the enucleation flasks by trypsini-

zation and scraping and then replated onto fresh dishes. Trypsin treatment was shown to remove nearly all surface proteins iodinatable by LPO when labeling of the cytoplasts was done within 10 min of trypsinization. Reattachment of the cytoplasts was monitored microscopically and surface labeling was done at various times. It may be seen (Fig. 6) that iodinatable fibronectin is not detectable on the cytoplast up to 10 h although other labeled proteins rapidly reappear. When, after 1 h, attached cytoplasts (Fig. 6 *b*) are compared to those still in suspension (Fig. 6 *a*), some differences are found. Labeling of the 87 and 75-kd proteins is greatly enhanced in the attached cytoplasts and a protein at 29 kd, heavily labeled in the attached cytoplasts, is not detectable in those in suspension. Interestingly, although no labeled fibronectin is detectable in the attached species, a trace amount is found on the cytoplasts in suspension. Longer incubation (3–4 h) of these suspended cytoplasts resulted in the attachment of most (>75%) to the substratum (Fig. 6 *c*). Note that iodination after 10 h of incubation (Fig. 6 *d*) labels a protein of ~102 kd on the cytoplast surface. This protein may be identical to the 100-kd protein detected on intact cells.

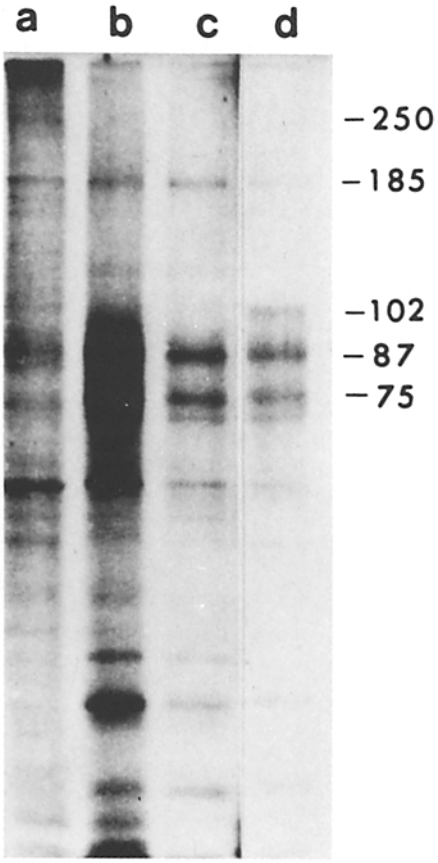

FIGURE 6 Reappearance of surface proteins on cytoplasts after trypsinization. Cytoplasts were removed from the polylysine treated substratum by trypsin treatment (0.05%) for 15 min followed by scraping and then incubated in fresh medium on new culture plates. After 1 h, ~50% had reattached. The two subpopulations (attached and suspended) were LPO iodinated separately. Lane *a* represents the autoradiogram of the ^{125}I labeled cytoplasts that were still in suspension, lane *b* represents the labeling pattern of the cytoplasts which had reattached to the new surface. Lane *c* is the pattern of label given by cytoplasts incubated for 4 h before iodination and lane *d* from cytoplasts incubated for 10 h. In the latter two samples, ~75% of the cytoplasts had reattached and only these were labeled.

It was considered that the increased labeling of the 75 and 87-kd cytoplast proteins might result from exposure to the cytochalasin B rather than enucleation. Treatment of whole cells with cytochalasin B for 30 min followed by incubation for up to 35 h in fresh medium resulted in no change in the pattern of labeled proteins compared to untreated cells (data not shown). Thus, it seemed that this was not likely.

DISCUSSION

At least six different proteins were judged to be exposed on the cell surface of chick embryo fibroblasts by the criterion of lactoperoxidase iodination. Three of these six (250, 87, and 75 kd) may be identical to those described by Stone et al. (29) as the major membrane proteins of the CEF. Those authors, however, were unable to label any but the 250-kd species by LPO iodination using carrier-free ^{125}I. The apparent K_m of lactoperoxidase for iodide is in the range of 1–10 μM (21) under the labeling conditions and the inclusion of carrier iodide at this concentration appears to be essential iodination unless large amounts (>500 μCi/ml) of ^{125}I are used. Concentrations in excess of 10 μm should be avoided because of the possible production of free I_2 (20).

One major concern was that the iodinatable proteins were noncellular and perhaps derived from the serum. However, it was demonstrated that when cells were incubated with iodinated serum, no specific binding could be detected. After the cells were subjected to the washing procedure only a trace amount of labeled albumin remained bound. In addition, incorporation of [^{35}S]methionine into these proteins was demonstrated. These data indicate that the proteins are cell derived.

A second question was whether these iodinated proteins were intrinsic or extrinsic membrane proteins. This distinction, which is rather vague and difficult to establish, was approached by attempting to extract these proteins under a variety of conditions. It has been reported that hypertonic conditions or high pH can elute extrinsic proteins from membranes (12, 28). Conditions of high salt (0.5 M NaCl) and pH (0.007 M NaOH) failed to extract any of the iodinated proteins either from whole cells or isolated membranes. Fibronectin, which is an extrinsic protein (10), was, however, not extracted by these conditions. This is consistent with the findings of others (M. Imada, University of Colorado, personal communication). Resistance of this extrinsic protein to such conditions makes unequivocal identification of any of these proteins as intrinsic or extrinsic difficult. However, the failure of these conditions to remove these proteins strongly implies that they are membrane in nature and not simply adsorbed soluble or cytoskeletal components.

The major finding of this study is that iodinatable surface proteins do not distribute randomly between the membranes of the cytoplasts and karyoplasts during enucleation. This suggests that these proteins are somehow constrained in the plasma membrane. The possibility that these proteins were specifically associated with the substratum was eliminated by the demonstration that the material remaining with the substratum after detachment of iodinated cells by EGTA and centrifugation contained only small amounts of labeled fibronectin while the other surface proteins were undetectable. This indicates that these proteins are not directly associated with the substratum or present in a substratum-associated extracellular matrix. Fibronectin has been reported to exist in a fibrillar network on the normal CEF membrane (32), and may polymerize to form a high molecular weight complex (13). Hynes and

Destree (11) have reported the existence of extensive disulfide bonding on the cell surface, primarily involving fibronectin complexes. If the fibronectin network is anchored directly or indirectly to the substratum (10), these observations could account for the lack of fibronectin on the karyoplast membrane. The lack of effect of colchicine and vinblastine on the partition of surface proteins after enucleation strongly implies that the presence of intact microtubular structures is not a determinant (30) and, since cytochalasin B is present, intact microfilaments may not be required. Taken together, our data suggest that the intact cytoskeleton is not directly involved in the asymmetric partition of these surface proteins.

Inclusion of PMSF in the medium before and during enucleation decreased by only 10–20% the small quantity of radiolabel lost from the membranes and no qualitative differences in labeling patterns were seen upon SDS electrophoresis. Although some proteolysis may be occurring (possibly due to release of lysosomal proteases from fragmented cells), this cannot account for the absence of certain proteins from only the karyoplast membranes. Shedding from the karyoplast membrane after enucleation also did not appear to be a reasonable explanation for the lack of fibronectin since the same amount of fibronectin (10% of the total fibronectin) could be recovered in the medium independent of enucleation.

No replacement of fibronectin or changes in other proteins on the karyoplast surface could be detected up to 8 h after enucleation, indicating that the karyoplast membrane was relatively stable. This also provided further evidence that surface proteolysis of the karyoplast was not responsible for the observed differences. Failure of the karyoplasts to replace their surface proteins may be attributable to their lack of Golgi apparatus (27) or other components. Cytoplasts which had been trypsinized and replated also did not appear to replace surface fibronectin, but certain other surface proteins were fairly rapidly regenerated (Fig. 6).

Our observation that there is a nonrandom partition of LPO-iodinatable surface proteins between the cytoplast and karyoplast after cytochalasin B-induced enucleation suggests that some surface proteins must be anchored, in some fashion, within the plasma membrane of the normal cell. The nature of the constraint mechanism is not clear but it appears to be independent of the intact cytoskeleton since there is no sensitivity to microtubule and microfilament disrupting agents. Disulfide bonding between fibronectin and other surface proteins (11) could limit most proteins to the cytoplast during enucleation particularly if the fibronectin complex is bound to the substratum. Alternatively, a fibrillar network of substratum-associated fibronectin might also serve to trap otherwise free membrane proteins in its interstices.

This work was supported in part by a grant to J. A. Gordon from the National Institutes of Health (NIH), by grant no. CA 15823 from the NIH, and by a gift from R. J. Reynolds Industries, Inc. D. P. Witt is the recipient of Research Fellowship grant no. IF32 CA-06380 from the NIH.

Received for publication 4 March 1982, and in revised form 11 May 1982.

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