USE OF IMMOBILIZED LACTOPEROXIDASE TO LABEL L CELL PROTEINS INVOLVED IN ADHESION TO POLYSTYRENE

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ABSTRACT

Proteins involved in the attachment of murine L cells to polystyrene have been identified by a technique designed to iodinate only those macromolecules coming into closest apposition to the substratum. Whereas soluble lactoperoxidase (LPO) catalyzes the radioiodination of a broad spectrum of polypeptides, the same enzyme immobilized on polystyrene tissue culture flasks discriminately labels 55,000 and 42,000 mol wt polypeptides that adhere tightly to the substratum after the cells are removed. One-dimensional peptide mapping following limited proteolysis showed that the labeled 55,000 mol wt polypeptide is similar to a component of comparable molecular weight present in the detergent-extracted cytoskeleton. The functional association of two cytoskeletal structures, presumably 10-nm filaments and actin, is discussed, and alternative explanations for their susceptibility to iodination by immobilized LPO are presented.

Neoplastic cells were observed in early tumor biology studies (11) to have less intercellular adhesiveness than normal cells. As the quality of in vitro systems improved, this decreased adhesiveness to inert substrata became a cardinal feature of the transformed phenotype (6, 40, 41).

Recognizing the importance of cell-substratum interactions in determining cell morphology and behavior, workers have used various approaches to identify the proteins or other cell-surface components involved in attachment. For example, the polypeptides iodinated by lactoperoxidase (LPO) in cells in suspension have been compared with those labeled in attached cells (23). Other workers have examined the macromolecules remaining attached to the substratum when cells are removed, hypothesizing that at least some of these substances must be involved in attachment (12, 13, 36). More recently, cell surface proteins (14, 46, 48), and antisera directed against them (14, 46), have been examined for their ability to block or otherwise modify the attachment process.

These approaches were rather indirect inasmuch as no available method was capable of discriminately labeling only those proteins in the attachment regions of intact cells. Reasoning that the cell surface comes into closest apposition to the substratum in such regions, we devised an enzymatic probe capable of labeling cell surface proteins but constrained to act only at attachment sites. Thus, this report describes the radioiodination of certain proteins in intact murine L cells with LPO covalently coupled to the polystyrene substratum to which the cells are attached (7). Because only a restricted set of the proteins is labeled by this procedure and because these proteins tightly adhere to the substratum after the cells are removed, we suggest that they are the proteins that come into closest apposition with the substratum and mediate the attachment of L cells to polystyrene.

Two polypeptides were significantly labeled by

the immobilized LPO. The most intensely labeled band had an apparent molecular weight of 55,000 in SDS polyacrylamide gels and was consistently iodinated. Its peptide map, prepared by limited proteolysis and polyacrylamide gel electrophoresis (10), was shown to be similar to that of a 55,000 mol wt component of detergent-extracted cytoskeletons prepared by the method of Hynes and Destree (25). A cytoskeletal component of this molecular weight is present in a wide variety of cultured cells and is thought to be a component of 10-nm filaments. The minor band had the electrophoretic mobility of actin and was labeled to different extents in the two L cell sublines examined. Considering that the 10-nm filament subunit and at least some of the cellular actin may exist as a complex (22, 28), we propose several alternative relationships with the plasma membrane that would allow the direct interaction of cytoskeletal components with the substratum during cell attachment and spreading.

MATERIALS AND METHODS

Polystyrene tissue culture flasks (25 cm^2) and bacteriological petri dishes were obtained from Falcon Labware, Div. of Becton Dickinson & Co., Oxnard, Calif. and from Corning Glass Works, Science Products Div., Corning, New York. All chemicals were reagent grade. Dimethyl suberimidate dihydrochloride (DMS) was obtained from Pierce Chemical Co. Rockford, Ill. Dulbecco's modification of Eagle's minimum essential medium (DMEM) and fetal calf serum were purchased from Grand Island Biological Co., Grand Island, New York. Pronase (protease from *Streptococcus griseus*, type VI) was obtained from Sigma Chemical Co. St. Louis, Mo. and *Staphylococcus aureus* V8 protease from Miles Laboratories, Elkhart, Ind. Carrier-free Na[125]I was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, New York. Radioactivity was determined in a Packard Autogamma Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.).

The polystyrene to which the DMS was coupled (DMS-polystyrene) and the DMS-polystyrene to which the LPO was coupled (LPO-DMS-polystyrene) were prepared as previously described (7).

Cell Culture

The two lines of murine L cells used in these experiments were L929, obtained from Microbiological Associates, Walkersville, Md., and A9, obtained from Dr. Joseph Lucas, State University of New York, Stony Brook. Both lines were maintained in DMEM that contained 10% fetal calf serum and were checked monthly for *Mycoplasma* contamination by the bisbenzamide staining method described by Chen (5). The cultures were routinely subcultivated by Pronase dissociation. However, cells to be used for radioiodination either were grown in plastic bacteriological petri dishes to which they do not attach or were detached from tissue culture flasks by 12 mM lidocaine hydrochloride (Astra Pharmaceutical Co., Worchester, Mass.) in DMEM that contained 10% fetal calf serum. After they were

harvested, cells were washed with either phosphate-buffered saline (PBS) or DMEM and plated onto the appropriate surface at a density of 2 × $10^5/cm^2$ in DMEM without serum. Flasks were incubated at 37°C for 3 h or longer, which allowed the cells to attach and spread. Then radioiodination was performed as described below. Even though 5–10% of the cells plated were dead, no dead cells appeared to attach. When the attached cells were recovered by trypsinization, the viability was 100% by trypan blue exclusion.

Phase-Contrast Microscopy

L929 cells were harvested from suspension cultures growing on bacteriological petri dishes, washed in serum-free DMEM, and plated in the absence of serum onto the various substrata: ordinary tissue culture polystyrene, DMS-polystyrene, and LPO-DMS-polystyrene. Incubation was carried out for 7 h at 37°C and in an atmosphere of 10% CO_2. Floating cells were decanted, and the monolayers were rinsed four times with PBS. All observations were made with a Zeiss Invertoscope D.

Radioiodination Procedures

Cells either in suspension or attached to a surface were labeled with the standard labeling solution that contained 100 μCi Na[125]I and 11.0 μg LPO in 1.0 ml of PBS. Hydrogen peroxide (1.5 mM in PBS) was added in 10 aliquots of 10 μl each at 20-s intervals, and incubation was carried out at room temperature for 15 min with occasional agitation. When cells attached to LPO-DMS-polystyrene were labeled, additional soluble LPO was omitted from the reaction system.

After labeling, cells were rinsed four times with PBS. Suspension cells were dissolved in 1% SDS, and cells attached to the surface were scraped off, centrifuged, and then dissolved. Any material remaining on the flask surface was removed with 0.2% SDS. All material was then heated at 100°C for 1 min after the addition of 2-mercaptoethanol (1% final concentration), and aliquots were taken for determination of TCA-precipitable radioactivity and for SDS polyacrylamide gel electrophoresis.

SDS Polyacrylamide Slab Gel Electrophoresis

Electrophoresis was performed in a 5–15% linear gradient slab gel, 0.3 cm thick, according to a modification of a previously published procedure (26). The gel buffer contained 0.19 M Tris-HCl (pH 8.8), 0.1% SDS and 0.002 M EDTA. The mixing chamber contained 15% acrylamide, 0.127% bisacrylamide, 3.2% sucrose, 0.02% (TMED), and 0.1% ammonium persulfate in gel buffer. The reservoir contained an equal volume of 5% acrylamide, 0.127% bisacrylamide, 0.0625% N,N,N',N'-tetramethylethylenediamine (TMED), and 0.1% ammonium persulfate in gel buffer. After polymerization, a spacer gel containing 3% acrylamide, 0.08% bisacrylamide, 0.1% SDS, 0.05% TMED, and 0.04% ammonium persulfate in 0.06 M Tris-HCl (pH 6.8) was layered on the separating gel. The electrode buffer contained 0.05 M Tris-HCl, 0.38 M glycine, 0.1% SDS and 0.002 M EDTA. Electrophoresis was performed at 50 mA per gel. Gels were stained with 0.25% Coomassie Brilliant Blue in 50% methanol–7% acetic acid, destained with 20% methanol–7% acetic acid, dried, and autoradiographed with DuPont Cronex medical x-ray film (E. I. DuPont de Nemours & Co., Wilmington, Del.). The modified gel formula described above permitted the 0.3-cm-thick gels to be routinely dried without cracking. Molecular weight

markers were cold-insoluble globulin (257,000), α-actinin (109,000), transferrin (81,000), bovine serum albumin (68,000), catalase (60,000), ovalbumin (43,000), and ribonuclease (13,700).

Subcellular Fractionation

Cells labeled with either soluble or immobilized LPO were suspended in 0.01 M Tris-HCl (pH 7.4) and Dounce homogenized by 50 strokes of the tight pestle. The homogenate was centrifuged at 30,000 g for 40 min, after which the resulting pellet and supernate were analyzed by SDS polyacrylamide slab gel electrophoresis and autoradiography as described above. Aliquots equal to 1/15 of the total cell homogenate and of each fraction were subjected to electrophoresis.

Isolation and Radioiodination of Microtubule and Detergent-Extracted Cytoskeleton Proteins

Once-polymerized microtubule protein was prepared from mouse brain homogenates by the method of Dentler, Granett, and Rosenbaum (15), except that Tris-HCl was used instead of PIPES. Brains were homogenized at 0°C in 1 mM MgSO₄, 2 mM EGTA, 1 mM GTP, 100 mM Tris-HCl, pH 6.9 (1.0 ml of solution per g wet wt of brain) and centrifuged at 130,000 g for 1 h at 4°C. The supernate was diluted with an equal volume of the same solution containing 8 M glycerol and incubated at 37°C for 30 min to assemble microtubules. These microtubules were then collected by centrifugation at 130,000 g for 1 h at 25°C, resuspended in the Tris-buffer described above at 0°C with a Dounce homogenizer with 1/3 the volume of the initial supernate, and incubated at 0°C for 30 min to depolymerize the microtubules. This solution containing once-polymerized microtubule protein was iodinated as described above. SDS polyacrylamide gel electrophoresis showed the preparation to consist predominantly of two well-separated bands of ~52,000 and 55,000 mol wt.

Detergent-extracted cytoskeletons were prepared by the method of Hynes and Destree (25). L929 cells were grown in suspension, pelleted, lysed with 1% Nonidet P-40 (Shell Chemical Co., Houston, Tex.), 50 mM Tris-HCl (pH 6.8), 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and centrifuged at 10,000 g for 10 min. The pellet was resuspended in PBS and iodinated as described above for cells in suspension. The iodinated material was washed four times in PBS by alternate resuspension and centrifugation at 10,000 g for 10 min. The SDS polyacrylamide gel pattern was essentially the same as that described by Hynes and Destree (25), with prominent bands at 42,000 and 55,000 mol wt.

Peptide Mapping by Limited Proteolysis and SDS Polyacrylamide Gel Electrophoresis

This procedure is a modification of the one described by Cleveland et al. (10). After electrophoresis as described above, preparative gels were stained for no more than 10 min with vigorous agitation and destained with cold water for 15 min. Bands of interest were cut from the gel, rinsed in a solution containing 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, and 1 mM EDTA, and were used immediately or stored at −20°C. 1.5-mm-thick and 15% acrylamide gels with 2-cm 3% stacking gels (40:1 by weight acrylamide:bisacrylamide) were used for the mapping. Because the gel slices were 3 mm thick, they could be trimmed to the size of the individual slots and used for two separate mapping experiments. The slices were pushed to the bottom of

the slot with a spatula and overlaid with an aliquot of Tris buffer that contained 20% glycerol to fill up the spaces around them. Then 10 μl of buffer that contained 10% glycerol and 0.25 μg of Staphylococcus aureus V8 protease was overlaid on each well. Electrophoresis was initially performed at 50 V/gel. The current was turned off for 30 min when the dye reached the bottom of the stacking gel. Finally, electrophoresis was resumed at 25 mA/gel. Staining and destaining of the gels were performed as usual, and autoradiography was performed as described above. To enhance the detection efficiency, an x-ray intensifying screen (Rädelin STF-2, U. S. Radium Corp., Brooklyn, N.Y.) was used and exposed at −70°C (27).

RESULTS

Morphology of Attached L Cells

Fig. 1 shows the phase-contrast micrographs of L929 cells 7 h after they were plated in the absence of serum onto the various experimental surfaces. Typically, cells that attached to tissue-culture polystyrene were spread, and assumed a fibroblastic morphology (Fig. 1 a). Some round cells were attached but not spread. It is likely that these were anchored by processes that inserted between the spread cells. Fig. 1 b and c shows cells that were attached to DMS-polystyrene and to LPO-DMS-polystyrene, respectively. It can be seen that the morphology of the cells on both surfaces is essentially similar to that of cells attached to unmodified polystyrene, despite the irregular, pitted appearance of the modified surfaces. Interestingly, the LPO-DMS-polystyrene surface does not appear as rough as the surface without LPO; however, this may merely reflect variation from one experiment to the other. Parallel flasks with cells scraped off did not show any recognizable cell fragments adhering to the substratum (not shown).

Electrophoretic Pattern of Substratum-bound Material (SBM)

Fig. 2, lane b, shows the typical distribution of polypeptides left attached to a polystyrene tissue-culture flask after the cells were scraped off. Materials from the modified surfaces yielded essentially identical electrophoretic patterns (not shown). To detect the SBM proteins by Coomassie Blue staining, the material was lyophilized and concentrated fivefold before being subjected to electrophoresis. As judged from the Coomassie Blue staining profile, the SBM consists largely of polypeptides with molecular weights ranging from 30,000 to 100,000, the major band being a 55,000 mol wt polypeptide. A 257,000 mol wt polypeptide that comigrated with cold-insoluble globulin is not
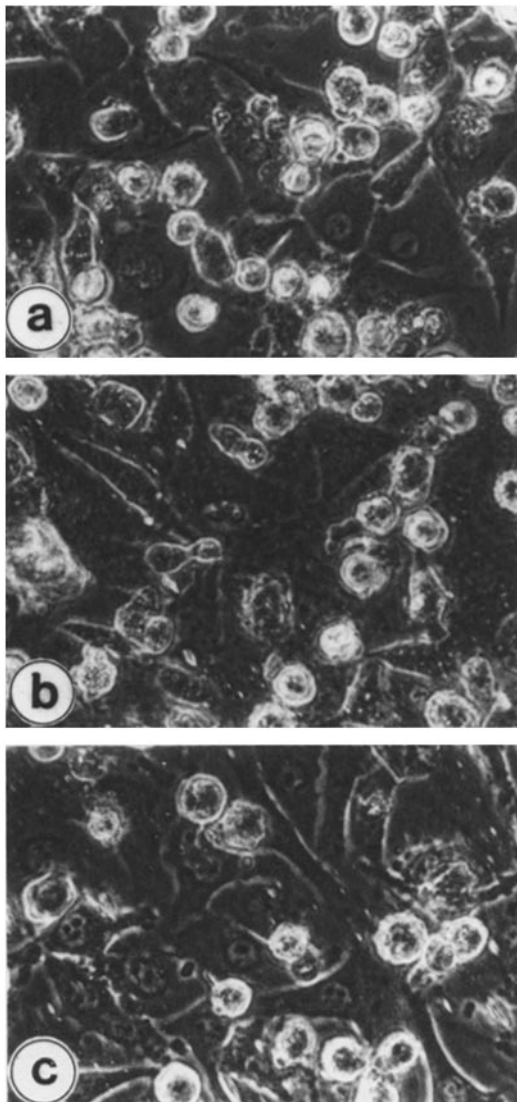
FIGURE 1 Phase-contrast micrographs of L929 cells attached to various substrata. Cells were plated in serum-free DMEM onto (*a*) ordinary tissue culture polystyrene; (*b*) DMS-polystyrene; and (*c*) LPO-DMS-polystyrene. Incubation was continued for 7 h at 37°C. × 128.

found in the SBM but, rather, remains cell associated. Protein determinations (31) showed that the SBM accounted for about 5% of the total cell proteins.

Patterns of Iodinated Polypeptides from L929 Cells

When soluble LPO was used to iodinate cells in suspension and cells attached to unmodified pol-

ystyrene or DMS-polystyrene, a substantial proportion of the incorporated radioactivity was found to be cell-associated, as is shown in lanes *c*, *d*, and *f* of Fig. 2. It should be kept in mind that even though the autoradiograms (Fig. 2, lanes *d–g*) show equal amounts of radioactivity in cells and in the SBM, the latter samples were concentrated fivefold before electrophoresis. In contrast, when immobilized LPO was used, no labeled bands could be detected in the cell-associated polypeptides; most of the radioactivity remained at the top of the gel, representing either aggregates that could not be dissociated with SDS and 2-mercaptoethanol or high molecular weight proteoglycans (Fig. 2, lane *h*). On the other hand, the SBM was preferentially iodinated by immobilized LPO, when compared with the cells.

The patterns of cell-associated polypeptides iodinated with soluble LPO are very similar, whether the cells were labeled in suspension or attached. This is probably so because the same polypeptides are labeled under both conditions and the SBM comprise only a small proportion of the total labeled peptides. The molecular weights of the labeled bands are listed in Table I. It is worth noting that a 257,000 mol wt band, probably LETS protein or fibronectin, is present but is not the only major labeled band. There are additional prominent bands with apparent molecular weights of 135,000–150,000, 55,000, and 30,000. A band of 42,000 mol wt is faintly labeled. Another faint band of 90,000 mol wt is not listed in Table I because it comigrates with labeled LPO.

The ^{125}I-labeling patterns of the SBM from the unmodified polystyrene (Fig. 2, lane *e*) and DMS-polystyrene (Fig. 2, lane *g*) differ from that of the cell-associated polypeptides (Fig. 2, lanes *d* and *f*) in that the high molecular weight bands are greatly reduced or absent. A 55,000 mol wt band is weakly labeled, whereas bands of 50,000 and 30,000 mol wt are intensely labeled.

The pattern of polypeptides labeled with LPO-DMS-polystyrene is dramatically different from all the others. In this case, the 55,000 mol wt polypeptide is labeled most intensely; less label is incorporated into the 50,000 and 30,000 mol wt bands. Thus, the conclusion to be drawn from the data presented up to this point is that a subset of the polypeptides iodinated by LPO remains attached to the various substrata, and that only a few of these come into sufficiently close proximity to the LPO-DMS-polystyrene surface for iodination to be catalyzed by the immobilized enzyme.
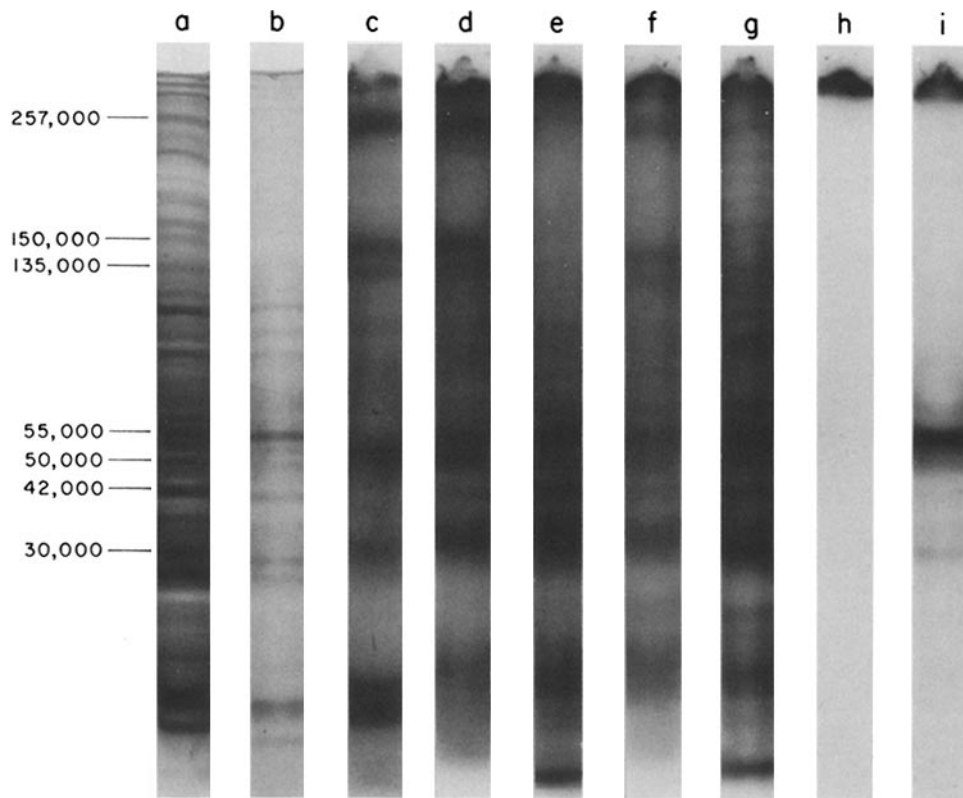
FIGURE 2   Analysis of iodinated polypeptides from L929 cells on 5–15% SDS polyacrylamide slab gels. After iodination with either soluble or immobilized LPO, the cells were scraped off, and the materials remaining on the flask were removed with 0.2% SDS. The latter were concentrated fivefold before electrophoresis. Coomassie Blue-staining profiles of (a) whole cells and (b) SBM. Autoradiograms of iodinated polypeptides from (c) whole cells labeled in suspension; (d) whole cells and (e) SBM labeled with soluble LPO following attachment to ordinary tissue culture polystyrene; (f) whole cells and (g) SBM labeled with soluble LPO following attachment of DMS-polystyrene; (h) whole cells; and (i) SBM labeled with LPO immobilized on the LPO-DMS-polystyrene surface to which the cells were attached.

Electrophoretic Pattern and Distribution of Iodinated Polypeptides from A9 Cells

As was the case with the L929 cells, the Coomassie Blue-staining profile or materials remaining on unmodified polystyrene (lane b of Fig. 3) after removal of A9 cells shows the predominance of a 55,000 mol wt polypeptide; this pattern is identical with that obtained from the modified polystyrene substrata (data not shown). Also not shown are the patterns of polypeptides labeled by soluble LPO, which were found to be similar whether the A9 cells were iodinated in suspension or attached. In contrast to the L929 cells, the LETS protein from A9 cells is absent or reduced when labeled with soluble LPO (Fig. 3, lane c). As for the distribution of radioactivity between the scraped cells and the SBM, it is similar to that seen in L929 (Fig. 3, lanes c and d).

With immobilized LPO, the radioactivity incorporated into SBM continues to be high when compared with the radioactivity remaining cell associated, but the difference is not as dramatic as for L929. In addition to the 55,000 mol wt polypeptide, a band comigrating with actin is significantly labeled with immobilized LPO (Fig. 3, lane f). The cell-associated polypeptides have the same labeling pattern as those remaining bound to the substratum, suggesting that, in this experiment, some of the SBM remained tightly associated with the scraped cells.

TABLE I

Molecular Weights and Distribution of Iodinated
Polypeptides from L929 Cells

| | Location of polypeptides | | |
| | Labeled by soluble LPO | | Labeled by im-mobilized LPO |
Molecular weight	Cells	Substra-tum	Substratum*
257,000	++	±‡	−
135,000–150,000	++	−	−
55,000	+	+	+++
50,000	++	+	+
42,000	±	+	±
30,000	++	+++	+
<30,000	++	+++	−

* Polypeptides remaining associated with the scraped cells were not significantly labeled.

‡ Labeling was either very faint or not seen in all experiments.

The symbols +, ++, and +++ refer to weak, moderate, and intense degrees of labeling, respectively.

Fractionation of Cells Labeled with Soluble and Immobilized LPO

Fig. 4 A shows the patterns of Coomassie Blue-staining polypeptides obtained upon fractionation of L cells labeled in suspension with soluble LPO. The separation of soluble from particulate elements appears to have been good because at least one protein (94,000 mol wt), which we know to be a soluble component of the cytoplasm (26), is found in the soluble fraction of this experiment. Labeling is confined entirely to the particulate fraction, strongly suggesting that the LPO has not penetrated beyond the plasma membrane. Other experiments, in which labeling was performed at 4°C to retard phagocytosis, yielded identical labeling patterns. Therefore, the labeled polypeptides are probably not components of phagocytic vacuoles. Fig. 4 B shows the patterns of stained and of labeled peptides obtained following fractionation of cells iodinated with immobilized LPO. Again, the soluble proteins are well separated from
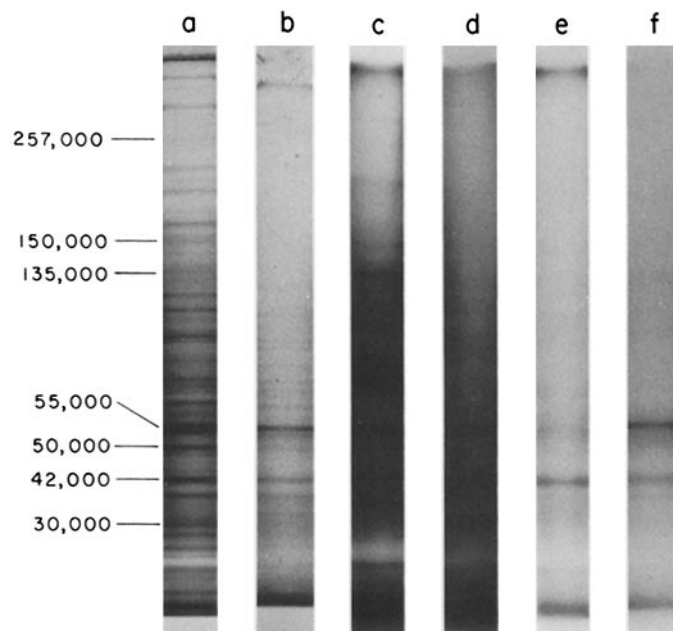

FIGURE 3 Analysis of iodinated polypeptides from A9 cells on 5–15% SDS polyacrylamide slab gels. After iodination with either soluble or immobilized LPO, the cells were scraped off, and the materials remaining on the flask were removed with 0.2% SDS. The latter were concentrated fivefold before electrophoresis. Coomassie Blue-staining profiles of (a) whole cells and (b) SBM. Autoradiograms of iodinated polypeptides from (c) whole cells labeled in suspension; (d) SBM labeled with soluble LPO following attachment to ordinary tissue culture polystyrene; (e) whole cells; and (f) SBM labeled with LPO immobilized on the LPO-DMS-polystyrene surface to which the cells were attached.
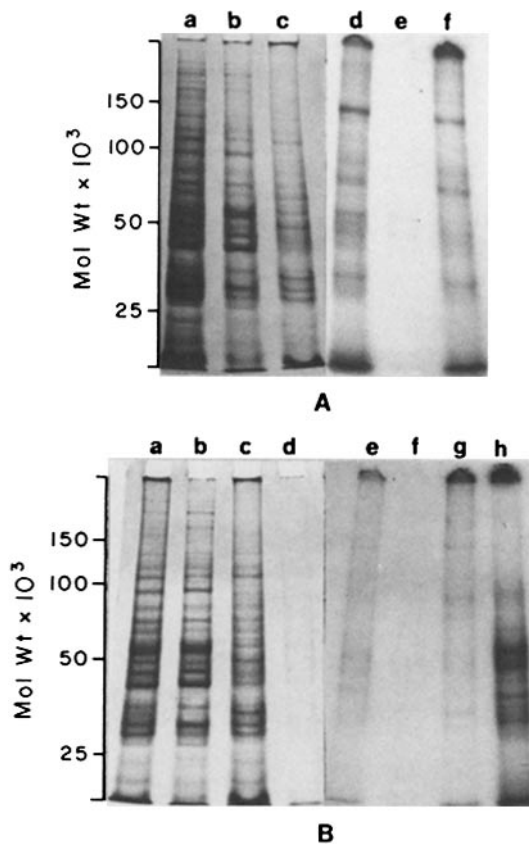
FIGURE 4 SDS polyacrylamide gel analysis of iodi-
nated polypeptides from L cell following subcellular
fractionation. (A) Cells iodinated in suspension using
soluble LPO. Coomassie Blue-staining patterns of (a)
total cell homogenate; (b) soluble protein fraction; (c)
particulate fraction; and (d–f) corresponding autoradi-
ograms. (B) Monolayer cells iodinated with immobilized
LPO. Coomassie Blue-staining patterns of (a) total cell
homogenate; (b) soluble protein fraction; (c) particulate
fraction; (d) SBM; and (e–h) corresponding autoradi-
ograms. Fractionation was performed as described in
Materials and Methods. Equal proportions of the total
cell homogenate and of each fraction were subjected to
electrophoresis.

the particulate elements. As described above, there
is no labeling of soluble proteins, and most of the
radioactivity is found in the SBM. This is so, even
though, in this experiment, the SBM were not
concentrated before electrophoresis.

Peptide Mapping by Limited Proteolysis and SDS Polyacrylamide Gel Electrophoresis

By using immobilized LPO, we significantly
iodinated a substratum-bound polypeptide with

an apparent molecular weight of 55,000 in murine
L cells. Because the two major classes of cellular
proteins comprising microtubules and 10-nm fila-
ments have components whose molecular weights
on SDS gels are ~55,000, it was deemed necessary
to compare their peptide maps with the map of the
labeled SBM polypeptide. Iodinated LPO was also
included in the mapping experiment because it
was conceivable that the labeled SBM polypeptide
could be an LPO degradation product. Bands of
interest: α-tubulin, β-tubulin, the 55,000 mol wt
component from detergent-extracted cytoskele-
tons, SBM 55,000 mol wt polypeptide, and LPO.
These bands were cut from preparative gels and
subjected to simultaneous digestion and separation
in a polyacrylamide gel as described in Materials
and Methods. Fig. 5 shows the autoradiogram of
the cleaved peptides from the various prepara-
tions. It can be seen that the SBM polypeptide
(Fig. 5, lane c) does not have a fragment pattern
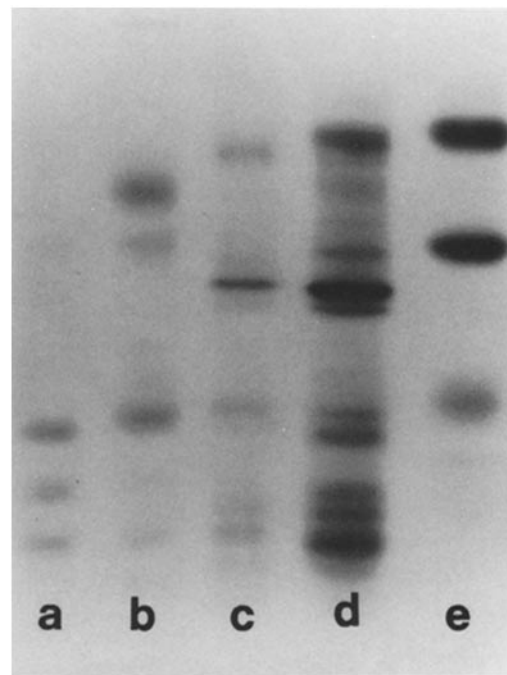that exactly matches any of the standards. How-

FIGURE 5 Autoradiogram of peptide maps obtained by
limited proteolysis and separation in a 15% SDS poly-
acrylamide slab gel. Gel slices containing the desired
polypeptides were reelectrophoresed in the presence of
0.25 μg of Staphylococcus aureus V8 protease: (a) mouse
brain α-tubulin; (b) mouse brain β-tubulin; (c) SBM
55,000 mol wt polypeptide; (d) 10-nm filament
subunit(s); and (e) LPO.

ever, each band in the SBM polypeptide pattern does have a corresponding band in the pattern obtained from the 55,000 mol wt component of detergent-extracted cytoskeletons (Fig. 5, lane d). We note that bands corresponding to the proteolytic fragments of α- and β-tubulin can also be found in the lane d pattern. On this basis, we conclude that the SBM polypeptide may be one of the components of detergent-extracted cytoskeletons.

DISCUSSION

Several factors were considered in designing our system. Firstly, labeling should be restricted to those proteins coming into direct contact with the enzyme. This requirement is satisfied by immobilized LPO because the enzyme must form an enzyme-substrate complex with exposed tyrosine residues in order to iodinate proteins (33). Secondly, labeling should be restricted to proteins in regions of cell-substratum adhesion. This is so because sites of cell-substratum attachment are generally regarded as regions of close proximity (50–100 Å) between the cell and substratum surfaces (review by Grinnell, reference 20), whereas regions of the plasma membrane not involved in adhesion are much farther away from the substratum. Finally, the LPO probe should be capable of

spanning the 50–100 Å gap between cell surface and substratum but incapable of penetrating through the plasma membrane to label internal proteins. This requirement is also satisfied because the combined length of the LPO molecule and the spacer arm is ~70 Å (7). Inasmuch as these requirements are all met, we expect that the proteins labeled when cells attach to LPO-polystyrene are, indeed, the mediators of adhesion under our experimental conditions.

Attachment sites occupy a small proportion of the total cell surface area (4, 21, 36) and have adhesive properties different from those of the remainder of the membrane (16). Judging from this, we expected that immobilized LPO would incorporate less radioactivity and label fewer polypeptides than the soluble enzyme, and indeed, this is what we found. Fig. 6 illustrates our conception of the relationship between a cell and the immobilized LPO that would result in the discriminate labeling of proteins in attachment regions.

Certain possible limitations of the technique, however, should be considered. For instance, the presence of LPO dimers or trimers coupled to the surface could lead to the iodination of proteins located farther away from the substratum. Alternatively, attachment of cells to polystyrene in regions between groups of LPO molecules could

$$\text{PROTEIN} + {}^{125}\text{I} \xrightarrow[\text{H}_2\text{O}_2]{\text{LPO}} \text{PROTEIN} - {}^{125}\text{I}$$
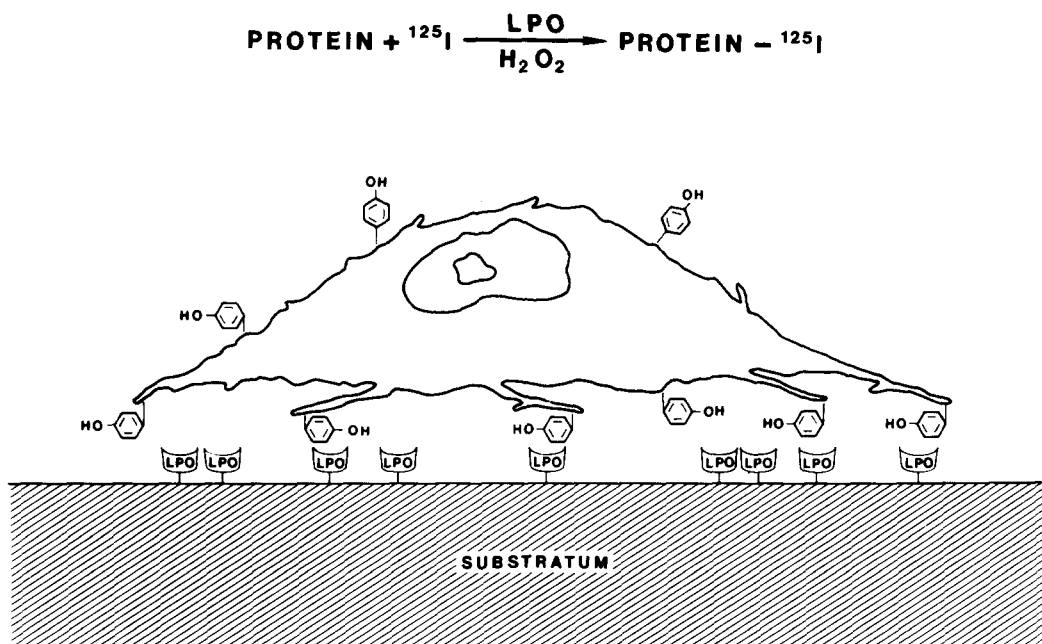

FIGURE 6   Schematic illustration of a cell attached to an LPO-polystyrene substratum.

result in iodination of proteins in the immediate vicinity of attachment regions but not directly interacting with the substratum. Even if one of these possibilities were true, however, we would still expect the immobilized enzyme to label only the restricted set of surface proteins found on the lower surface of attached cells.

Are the cells attaching to a film of LPO molecules or to polystyrene? The available evidence indicates that at the optimal DMS concentration (0.03 M) used for coupling, a monomolecular film of LPO molecules was not formed. In fact, we observed that more than twice as much enzyme can be coupled to the flask as the DMS concentration is raised beyond that used in our experiments, indicating that the amount of bound enzyme at the optimal concentration was below saturation (7). Therefore, we think that, under our experimental conditions, the cells are attached to a substratum having the characteristics of both LPO and polystyrene.

Electrophoretic Patterns and Distribution of Polypeptides Iodinated with Soluble LPO vs. Immobilized LPO

Using the conventional labeling technique, we found that of the 35 or more Coomassie Blue-stained bands in murine L cells, about 8 were heavily iodinated and 4 were faintly iodinated. The most intensely labeled bands have apparent molecular weights of 257,000 (comigrated with cold-insoluble globulin), 135,000–150,000, 55,000, 50,000, 30,000, and <20,000. Subcellular fractionation showed that labeling of soluble cytoplasmic proteins was negligible. When attached cells were iodinated by LPO in solution, their ^{125}I-labeling patterns were not significantly different from those of cells iodinated in suspension. This is in contrast to the finding of Hunt and Brown (23), who reported a broad spectrum of iodinated polypeptides with approximate molecular weights ranging from 25,000 to 250,000 when they labeled L cells in suspension and only two labeled bands, 250,000 and 80,000 when they labeled monolayer cells. Because they used cells that had been grown in the presence of serum and that were iodinated while they were attached to the original flasks in which they were grown, it is possible that some labeling of LPO and serum proteins accounts for their results. When we performed the experiments in a similar manner, a substantial amount of iodine was incorporated into a group of polypeptides that

comigrated with LPO, bovine serum albumin, and transferrin, i.e., in the 68,000–90,000 mol wt range.

In a typical iodination experiment, serum was omitted from the reaction mixture, and the cells were washed several times before labeling. Also, direct labeling of serum proteins (in the absence of cells) adsorbed to ordinary and to modified polystyrene did not result in iodination of polypeptides similar to those labeled in the cells or in the substratum (data not shown); in fact, serum transferrin and albumin comprised the major labeled bands. Likewise, labeling of nonviable cells did not appear to be a serious source of artifact because dead cells did not attach, and leakage of the highly insoluble 10-nm filament protein seems most unlikely (3, 25). Subsequent recovery by trypsinization from a control flask, just before labeling, showed that the attached cells were 100% viable as determined by trypan blue exclusion.

Iodination of attached cells was performed with cells plated onto the various substrata (ordinary polystyrene, DMS-polystyrene, and LPO-DMS-polystyrene [soluble LPO was omitted from the last system]). Because we were concerned with the adverse effect that the modified surface might have on cell attachment, the DMS-polystyrene labeling system was included as a control, and its pattern of iodinated polypeptides was compared with that of cells attached to unmodified polystyrene. The results of the comparison indicate that there is no difference between the labeling patterns of cells attached to ordinary polystyrene and those attached to DMS-polystyrene. Observation by phase-contrast microscopy showed that there is no apparent difference in the morphology of cells attached to the various surfaces. Labeling of attached cells by soluble LPO resulted in the incorporation of radioactivity into materials that, for the most part, remained cell associated. This is in contrast to labeling by the immobilized enzyme which radioiodinated fewer of the polypeptides that preferentially remained in the SBM after the cells were scraped from the LPO-DMS-polystyrene. Because the enzyme activities were approximately the same in both systems (soluble LPO and immobilized LPO), the difference in distribution of the labeled material can best be explained by the proposal that it results from a difference in the affinity of the labeled proteins for the substratum: the soluble LPO labeled proteins, which have low affinity for the substratum, are found on the exposed upper surfaces of cells and remain cell associated, whereas the immobilized enzyme labeled

proteins, which have high affinity for the substratum, are preferentially localized in regions of attachment and are more likely to remain substratum associated. This explanation is supported by the observation that the immobilized LPO labels fewer proteins than the soluble enzyme, and that those labeled by bound LPO remain preferentially associated with the substratum.

Interestingly, it was observed that LETS protein is not labeled by the immobilized enzyme, which suggests that it does not interact directly with the substratum. At first glance, this seems to contradict the suggestion that it is involved in cell-substratum adhesion. However, whereas the data of Mautner and Hynes (32) show that LETS protein is located under normal cells, they do not indicate whether it tightly adheres to the substratum. Reports that it is left on the substratum after the treatment of cell monolayers with detergents or chelating agents (3, 37) do not show that LETS protein directly mediates attachment. The fact that it restores the normal morphology and adhesiveness to transformed cells (1, 48) does not necessarily point to a direct interaction with the substratum. Other possibilities, such as interaction with the cytoskeleton or with other molecules on the upper surface of the cell, could also account for the observed effects. Despite the observation that LETS protein is not iodinated by the immobilized enzyme, an adhesive role for this glycoprotein in murine L cells is not completely excluded because there are convincing data showing that it mediates cell attachment to collagen (review by Yamada and Olden, reference 47).

It is worth noting that some of the polypeptides found in the SBM were not labeled by the immobilized enzyme. The possible explanations are: (1) that these proteins were there by virtue of their being complexed with other true adhesive molecules, (2) that they represent proteins that leaked out during the scraping after the iodination was complete, and (3) that they contained tyrosine residues that were not exposed. Although we do not have data to support any of these possible explanations, we favor the first possibility because of the prevailing concept that surface molecules do interact with the cytoskeleton and/or other surface components.

Identification of the Substratum-bound Polypeptides Labeled by Immobilized LPO

A 55,000 mol wt polypeptide that was consistently and predominantly iodinated by the immo-

bilized enzyme was mapped by the method of Cleveland et al. (10) and was shown to be similar to a component of the detergent-extracted cytoskeleton (25, 39). The possibility that these filaments might be involved in cell-substratum adhesion did not come as a total surprise to us because it is now gradually being recognized that these filaments interact with other components of the cytoskeleton, namely, microtubules and microfilaments (14, 19, 25, 43). Recently, Damsky et al. (14) obtained an antiserum that could bind to cell-surface components, thereby causing cells to round up and detach. Although this antiserum did not enter the cell, it caused a marked alteration in the distribution of 10-nm filaments, providing additional evidence that 10-nm filaments may be involved in attachment.

A 42,000 mol wt polypeptide with the mobility of muscle actin was intensely labeled in the A9 cells but variably labeled in the L929 subline. A similar protein (apparent mol wt 40,000–50,000) has been described in lymphocyte plasma membranes and has been shown to be iodinated in intact cells (42, 49). However, these studies indicate an antigenic relationship to either the H-2 or TL antigens, so molecular weight alone is not sufficient to identify the polypeptide labeled in our system as actin. If the labeled protein is actin, the simultaneous labeling of a component of 10-nm filaments and microfilaments would suggest a functional relationship between them. Other investigators have hinted at such an association between these two proteins on the basis of the following observations: they are found together in the material that remains substratum associated after the cells are removed (37) and in the Z disks of muscle cells (19, 28). Furthermore, they copurify from skeletal and smooth muscle cells (23, 28).

Despite these implications, the iodination of two intracellular proteins by a probe that is expected to label only surface proteins is perplexing. One satisfying explanation for our data is that both microfilaments and 10-nm filaments, at some point during the attachment process, may span the plasma membrane at sites of cell-substratum attachment. This hypothesis is indirectly supported by many previous studies. Both types of filaments are shown by electron microscopy to lie near the plasma membrane in attachment regions (18, 24, 30, 45) and are occasionally observed to insert into the plasma membrane (2, 9, 17, 29). Evidence for a functional interaction between microfilaments and the plasma membrane comes from the corre-

lation of the reduced adherence of transformed cells with the reduced amounts of plasma membrane-associated actin (35, 38, 44). Furthermore, both microfilaments and 10-nm filaments have been found in concanavalin A-induced caps (8), a structure in which transmembrane interactions must occur (34). On the other hand, the possibility that isolated microfilament and 10-nm filament subunits exist on the external surface of the plasma membrane cannot be completely discounted. It is conceivable that these isolated subunits can migrate along the lateral plane of the plasma membrane to aggregate at potential sites of attachment where subsequent interactions with a membrane-associated (inner surface) substance(s) occur, thus strengthening the force of adhesion and allowing cell spreading to proceed.

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