USE OF IMMOBILIZED LACTOPEROXIDASE TO LABEL MURINE FIBROBLAST PROTEINS INVOLVED IN ADHESION TO POLYSTYRENE

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SUMMARY

Proteins involved in the attachment of murine embryo fibroblasts to polystyrene have been identified by a technique designed to iodinate only those macromolecules coming into closest apposition to the substratum. Lactoperoxidase (LPase) covalently bound to the surface of the culture flask labelled a subset of substratum-bound polypeptides with a 42,000 M₉ species being most heavily labelled. Fibronectin was not labelled by this method. Soluble LPase, on the other hand, iodinated a wide range of polypeptides in cells attached to ordinary tissue culture polystyrene. Many of these polypeptides, including fibronectin, were cell-associated after scraping; however, bands of 50,000-55,000 and 42,000 M₉ remained bound to the substratum. The effect of serum was investigated and the results suggested that serum components blocked labelling of the 42,000-55,000 M₉ species by soluble LPase, but did not abolish labelling of similar polypeptides by the immobilized enzyme. The identity of the prominently labelled bands is discussed in the light of a functional interaction between two polypeptides, probably 10 nm filament protein subunits and actin, at sites of cell–substratum attachment.

INTRODUCTION

The association of cytoskeletal components with the plasma membrane has been reported by various investigators using transmission electron microscopy (Goldman, 1975; Lloyd, Smith, Woods & Rees, 1977), interference-reflection microscopy (Wehland, Osborn & Weber, 1979; Heath & Dunn, 1978), immunofluorescence (Wehland et al. 1979) and subcellular fractionation (Wickus, Gruenstein, Robbins & Rich, 1975). It is generally believed that this interaction is important in such normal cell behaviour as locomotion, adhesion, mitosis and surface molecule migration. Previous studies (Wickus et al. 1975; Pollack, Osborn & Weber, 1975; Rubin, Warren, Lukeman & Clements, 1978) have shown that transformed cells have reduced amounts of plasma membrane-associated actin, which may be correlated with the alterations in morphology and adhesive properties of these cells. Recently, two separate laboratories reported the presence of the src gene product in adhesion plaques (Rohrschneider, 1980) and its association with cytoskeletal structures (Burr, Dreyfuss, Penman & Buchanan, 1980). This is indirect evidence that the src gene product may alter cell adhesion via its influence on the cytoskeleton.

The major stumbling block in the study of cell–substratum interaction is the lack of a direct biochemical approach, which can identify the so-called 'attachment proteins'. As reported previously (Chin & Lanks, 1977, 1980), we have devised a
novel method using lactoperoxidase (LPase) immobilized onto the substratum to iodinate those polypeptides of monolayer cells that come directly into closest contact with it. The polypeptides iodinated by this method were tightly adherent to the substratum and their peptide maps showed a major band that was similar to that of a component of intermediate filaments (Chin & Lanks, 1980; Lanks & Chin, 1981).

In this report, we have extended our studies to normal mouse embryo fibroblasts (MEF). As previously seen, the immobilized enzyme discriminated a subset of the polypeptides labelled by the soluble enzyme. Again, labelling was restricted to polypeptides tightly adherent to the substratum. Fibronectin was not labelled by this method, although it was labelled by the soluble enzyme and remained cell-associated. In contrast to mouse L cells and macrophages, the band most prominently iodinated by immobilized LPase has an apparent molecular weight ($M_r$) of 42,000 and comigrates with muscle actin. Several possibilities are presented to explain why this known intracellular protein is labelled by an external probe. Control experiments showed that the same cellular proteins continued to interact with immobilized LPase in the presence of serum.

MATERIALS AND METHODS

Cell culture

Mouse embryos were dissociated proteolytically according to the method of Gwatkin (1973). Cultures were maintained in Dulbecco's modification of Eagle's minimum essential medium (DMEM) containing 10% foetal calf serum (Gibco, Grand Island, N.Y.) and incubated in 10% carbon dioxide at 37°C. Secondary or tertiary cultures were harvested with 0.0625% (w/v) Streptomyces griseus protease, type VI (Sigma Chemical Co.), washed with Dulbecco's phosphate-buffered saline (PBS) and plated on to the various substrata at a density of $2.5 \times 10^5$ cells/cm$^2$ in DMEM with or without serum. Polystyrene flasks (Corning, N.Y.) to which dimethylsuberimidate dihydrochloride (Pierce Chemical Co., Rockford, Ill.) was coupled (DMS-polystyrene) and DMS-polystyrene to which lactoperoxidase was coupled (LPase-DMS-polystyrene) were prepared as previously described (Chin & Lanks, 1977). The flasks were incubated overnight at 37°C in 10% carbon dioxide allowing cells to attach and spread as well as to recover from the effects of treatment with protease.

Radioiodination procedures

Mouse embryo fibroblasts (MEF), either in suspension or attached to a substratum, were labelled using the standard labelling system that contained 100 $\mu$Ci Na$^{125}$I (Schwarz/Mann, Orangeburg, New York) and 1.0 $\mu$g LPase (enzyme activity equivalent to that of immobilized LPase) in 1.0 ml of PBS. Hydrogen peroxide (15 mM in PBS) was added in 10 aliquots of 10 $\mu$l each at 20-s intervals and incubation carried out at room temperature for 15 min with occasional agitation. When cells attached to LPase-DMS-polystyrene were labelled, soluble LPase was omitted from the reaction system.

After labelling, cells were rinsed four times with PBS. Suspension cells were dissolved in 1% sodium dodecyl sulphate (SDS), whereas monolayer cells were scraped off, centrifuged and then dissolved in 1% SDS. Any material remaining on the flask surface was removed with 0.2% SDS. All material was then heated at 100°C for 3 min after the addition of 2-mercaptoethanol (1% final concentration) and aliquots were taken for determination of trichloroacetic acid-precipitable radioactivity and SDS/polyacrylamide gel electrophoresis. Radioactivity was determined in a Packard Autogamma Scintillation Spectrometer. Fig. 1 shows the sequence of steps in a typical iodination experiment.
**Fibroblast adhesion proteins**

Harvest cells by protease treatment

Wash 3x with serum-free DMEM

Resuspend in PBS

Iodinate in suspension

Wash 4x with PBS

Resuspend in PBS

Plate in DMEM ± serum on to:

- unmodified polystyrene or
- LPase-DMS-polystyrene

Incubate overnight at 37°C

Iodinate

Rinse 4x with PBS

Scrape into PBS

Centrifuge 5000 , 10 min

Cells

Substratum-bound material (SBM)

Lyophilize

Heat in the presence of SDS and 2-mercaptoethanol

Trichloroacetic acid precipitation and radioactivity determination

SDS/polyacrylamide slab gel electrophoresis

Autoradiography

Fig. 1. Sequence of steps in a typical iodination experiment.

**SDS/polyacrylamide slab gel electrophoresis and autoradiography**

Electrophoresis was performed in a 5% to 15% linear gradient slab gel, 0.3 cm thick, according to a modification of a previously published procedure (Chin & Lanks, 1980; Kasambalides & Lanks, 1979). 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 using Dupont Cronex medical X-ray film. The modified gel formula described above permitted the 0.3 cm thick gels to be dried routinely without cracking. Molecular weight markers were α-actinin (109,000), transferrin (81,000), bovine serum albumin (68,000), catalase (60,000), ovalbumin (43,000) and ribonuclease (13,000).

About 50 μg of cell protein was loaded into each well with the exception of the substratum-bound material, which was concentrated fivefold and loaded to the extent that it was available.
RESULTS

Electrophoretic patterns of polypeptides iodinated using soluble and immobilized lactoperoxidase

When MEF cells were labelled in suspension in the absence of serum using soluble LPase, a set of iodinated polypeptides was resolved as shown in Fig. 2 (lane c). Two major labelled polypeptides have molecular weights of 135,000–150,000 and there are minor bands in the 200,000 and 42,000–60,000 \( M_r \) regions. The 257,000 \( M_r \) polypeptide (comigrating with cold-insoluble globulin) seen in the Coomassie blue staining profile of whole cells (lane a) was not iodinated. In contrast, when attached cells were labelled using soluble LPase (lane d), bands in addition to the ones already mentioned were iodinated. These have apparent molecular weights of 257,000 and 109,000. Furthermore, labelling of the 55,000 and 42,000 \( M_r \) bands was definitely increased in intensity. A diffuse band of 94,000 \( M_r \) was variably labelled, but will not be discussed further since it comigrated with LPase. Fig. 2 (lane e) shows the electrophoretic pattern of substratum-bound material (SBM) obtained after attached cells were labelled with soluble LPase and then scraped off. The species are fewer and generally of lower \( M_r \) with prominent bands in the region of 55,000 to 42,000 \( M_r \). The 257,000 \( M_r \) band is not found in the SBM but, rather, remains entirely cell-associated.

When LPase, immobilized on the LPase-DMS-polystyrene substratum on to which the cells were plated, was used to iodinate MEF in the absence of serum, the distribution of radioactive materials was different from that obtained when soluble LPase was used to label attached cells. Fig. 2 (lane f) shows that little or no radioactivity was found to be cell-associated. Instead, the iodinated polypeptides were found mainly in the SBM and consisted of species at or below 60,000 \( M_r \) (lane g). Among these, a polypeptide with an apparent \( M_r \) of 42,000 was most prominently labelled. Again, no high \( M_r \) species in the SBM were iodinated even with the immobilized LPase. As judged from the Coomassie blue staining patterns of scraped cells and SBM (lanes a and b, respectively), relatively little protein was present in the SBM even though the amount of incorporated radioactivity markedly exceeded that found in the scraped cells.

Whereas only 16% of the total trichloroacetic acid-precipitable radioactivity incorporated by soluble LPase remained substratum-bound, all of that incorporated by immobilized LPase was found in the SBM. These data support the contention that immobilized LPase preferentially labels proteins in intimate contact with the substratum and not those in other regions of the cell surface. Densitometry of the autoradiograms showed that there was no increase in the relative labelling intensity of the 42,000 or 50,000–55,000 \( M_r \) bands in lane e as compared with lane d (Fig. 2). Thus, the difference in labelling pattern between SBM and cell-associated material is due mainly to decreased representation of several high molecular weight bands rather than to an absolute increase in labelling of the 42,000–55,000 \( M_r \) region. Similar analysis of the material labelled by immobilized LPase (lane g) showed that there was no increase in the relative labelling intensity of the 50,000–55,000 \( M_r \) region and
Fig. 2. SDS/polyacrylamide gel electrophoresis of MEF polypeptides iodinated in the absence of serum. After iodination with either soluble or immobilized LPase, the cells were scraped off and the material remaining on the flask was removed with 0.2% SDS. The latter were concentrated fivefold before electrophoresis. Coomassie blue staining profiles of scraped cells (a) and SBM (b). Autoradiograms of iodinated polypeptides from: c, whole cells labelled in suspension; d, scraped cells; and e, SBM obtained from cells labelled with soluble LPase following attachment to ordinary tissue culture polystyrene; f, scraped cells; and g, SBM obtained after labelling with LPase immobilized on the LPase-DMS-polystyrene surface to which the cells were attached.

only a 1.9-fold increase in the relative labelling intensity of the 42,000 $M_r$ band as compared with lane e. Since the total incorporation into SBM by immobilized LPase (lane g) was 2.2-fold lower than that into SBM by the soluble enzyme, the absolute labelling of the 42,000 $M_r$ band was almost exactly the same under both conditions, and differences in appearance of the autoradiograms are again due mainly to decreased labelling of bands outside the 50,000-55,000 and 42,000 $M_r$ regions.
Effects of serum components on the iodination patterns

To determine whether the iodinated polypeptides were serum derivatives or not, we added foetal calf serum (10% final concentration) to the plating medium and allowed the cells to attach to and spread on the appropriate substrata. At the end of the incubation period, the monolayers were rinsed with PBS and iodinated using either soluble or immobilized LPase as above. Control iodinations with serum in DMEM and without cells were performed. Fig. 3 (lane a) shows that in the absence of cells, serum components adsorbed to the surface of the flask and were iodinated by the soluble enzyme. Most of the radioactivity was found in a diffuse band having an apparent $M_r$ range of 60,000 to 80,000, presumably consisting of transferrin and albumin. Labelling of serum alone with the immobilized enzyme yielded similar results (data not shown). Incubating the cells with 10% foetal calf serum followed by iodination using soluble LPase revealed labelling patterns of the scraped cells and of the SBM as shown in lanes b and c, respectively. In contrast to Fig. 2 (lane d) the presence of serum proteins appeared to block the incorporation of iodine into some of the surface components of scraped cells (Fig. 3, lane b). Similarly, the SBM polypeptides (Fig. 3, lane c) were not appreciably labelled, except for a band corresponding to the $M_r$ of albumin.

When immobilized LPase was used to iodinate cells that had been incubated in the presence of serum, the scraped cells, as usual, were not found to contain much radioactivity (Fig. 3, lane d). The SBM (Fig. 3, lane e) contained heavily labelled bands corresponding to the $M_r$ of transferrin and albumin. In addition, two other discrete bands bearing apparent $M_r$ values of 42,000 and 30,000 were found.

DISCUSSION

These experiments using the MEF system were designed to answer two important questions raised in relation to previous studies using L cells. Normal cells were used to ensure that the attachment mechanism being studied was not unique to L cells, a line extensively adapted to cell culture conditions. In addition, controls employing attachment in the presence of serum were required in order to determine whether the cellular proteins, previously seen to be exposed to the substratum in serum-free medium, were still labelled by immobilized LPase under physiological conditions. As discussed in detail below, the results appear to indicate that cytoskeletal components do come into close approximation with the substratum when normal fibroblasts attach to polystyrene in the presence of serum.

The labelling patterns of L cells were previously found to be very similar regardless of whether they were in suspension or attached to a substratum (Chin & Lanks, 1980). In the MEF system, however, several additional bands were labelled in the attached cells. Considering that the L cells were not treated with proteolytic enzymes whereas the MEFs were, the difference can probably be accounted for by resynthesis of surface constituents in the interval between labelling of the suspension and attached cells. It is worth noting that when attached cells were labelled using soluble
LPase, all of the bands corresponding to those labelled in suspension remained cell-associated after scraping. In contrast, only some of the new bands remained cell-associated, e.g. fibronectin, while other bands were distributed between cells and substratum.

It is also noteworthy that LETS protein (fibronectin), which is present on the cell surface and accessible to iodination by soluble LPase, was not labelled by the immobilized enzyme. Therefore, this protein does not appear to play a direct role in the interaction of MEFs with a polystyrene substratum either in the presence or in the absence of serum. This finding is consistent with recent work suggesting that fibronectin is not present in regions of closest cell-substratum contact (Chen & Singer, 1980; Avnur & Geiger, 1981) and that it may not, in fact, be required for

The major substratum-bound polypeptides of 55,000 and 42,000 Mr, especially the latter, are preferentially labelled when cells are iodinated by immobilized LPase. We interpret this to mean that the proteins labelled by the immobilized enzyme are those having the highest affinity for the substratum. As such, they are also the surface proteins most likely to be directly involved in adhesion to polystyrene. The effect of adding serum during attachment was to eliminate or, at least, greatly reduce labelling of the 55,000 and 42,000 Mr species by soluble LPase. At the same time, a 68,000 Mr band comigrating with bovine serum albumin appeared in association with the substratum. These data suggest that serum components block labelling of attachment proteins by soluble LPase. Since these proteins are still accessible to immobilized LPase in the presence of serum, it seems reasonable to conclude that serum components are excluded from the regions of closest cell–substratum contact. In view of this finding, serum fibronectin may be merely one example of a protein that is removed from the substratum during attachment.

Previous work on L cells (Chin & Lanks, 1980) and a more extensive peptide mapping study of the L cell and macrophage polypeptides labelled by immobilized LPase (Lanks & Chin, 1981) indicate that the 55,000 Mr component labelled under identical conditions is a surface molecule, which remains in continuity with the cytoskeleton following non-ionic detergent extraction. Although 10 nm filaments were not isolated as such in these studies, a great deal of data from other laboratories (Bachvaroff, Miller & Rapaport, 1980; Geiger & Singer, 1980; Hynes & Destree, 1978) leave no doubt that they are the source of the 55,000 Mr polypeptide. Assuming that the 55,000 Mr polypeptides iodinated in L cells, macrophages and MEF are related, then we may conclude that the role of 10 nm filaments is greatly reduced in MEF attachment as compared with the cell types previously studied.

In contrast to the L cell and macrophage systems in which the 42,000 Mr polypeptide is a relatively minor species, it is the component most heavily labelled in MEFs attached to LPase-DMS-polystyrene. In addition, the number of labelled bands in the SBM is increased in MEFs as compared to the other two systems. The spectrum of proteins interacting with immobilized LPase appears to depend on cell type and could be due either to decreased labelling in the 50,000–55,000 Mr region or to increased labelling of the 42,000 Mr band. Our experience has been that the specific activity as well as the absolute degree of labelling by immobilized LPase is consistently lower in the MEF system as compared to either L cells or macrophages. This observation, albeit qualitative, is consistent with decreased labelling of the 50,000–55,000 Mr region in attached MEFs. The relatively greater number of labelled bands in MEFs can also be explained on this basis because longer exposure times were required to yield acceptable autoradiograms. Longer exposures would most likely have revealed similar minor bands if they had been used in previous experiments.

Both migration in SDS/polyacrylamide gels and preliminary peptide mapping data indicate that the 42,000 Mr polypeptide is identical to cytoskeletal actin, so
we have tentatively identified it as such. This would also be consistent with the observations that both 10 nm filaments and microfilaments insert into the plasma membrane in attachment regions (Wehland et al. 1979; Lazarides, 1980; Starger, Brown, Goldman & Goldman, 1978), and that the subunits of these filaments co-purify from skeletal and smooth muscle cells (Brecher, 1975). Therefore, the present study provides additional data to support the concept of a functional interaction between these two cytoskeletal components at sites of cell-substratum adhesion.

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REFERENCES


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