Identification of Cytoskeletal Components Involved in Attachment of L929 Cells and Macrophages to Polystyrene

KARL W. LANKS and NENA W. CHIN
Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203, and Department of Pathology, College of Physicians and Surgeons, Columbia University, New York 10032

ABSTRACT
We have previously shown that lactoperoxidase (LPO) covalently coupled to polystyrene tissue culture flasks can be used to radioiodinate monolayer cell proteins that come into intimate contact with the LPO-polystyrene surface. These studies have now been extended to include a detailed examination of the class of iodinated polypeptides migrating with apparent molecular weights of 50,000 and 55,000 in SDS polyacrylamide gels. Whereas in cultured L929 cells the 55,000 band is predominantly iodinated, in thioglycolate-activated murine peritoneal macrophages the 55,000 and 50,000 bands are of equal intensity. It is possible that the marked degree of exposure of the 50,000 mol wt polypeptide to immobilized LPO is related to the unique strength of macrophage attachment. After labeling of both L929 cells and macrophages with immobilized LPO, all polypeptides in this molecular weight region were subjected to peptide mapping by simultaneous limited proteolysis and electrophoresis in a second SDS polyacrylamide slab gel. The results clearly show that the two major polypeptides in this region are identical within the limits of resolution of this technique. The 55,000 mol wt polypeptide can also be identified in Triton X-100 cytoskeletons from L929 cells after labeling with soluble LPO either before or after detergent lysis. We conclude that this cell surface polypeptide is in continuity with the cytoskeleton and is preferentially exposed to the substratum during attachment to polystyrene.

It has become increasingly apparent that many cellular activities including locomotion, attachment to inert substrata, and movement of surface receptors involve the interaction of internal cytoskeletal structures with external membrane components. Thus, actin filament bundles and α-actinin have been consistently found in association with focal adhesion points (8, 11–13, 15, 18), while lectin- or antibody-induced clusters of cell surface components are seen to overlie stress fibers containing actin, myosin and α-actinin (6, 7). Although the precise relationship of fibronectin to the cell surface remains to be determined, ultrastructural studies suggest that it may be in direct continuity with microfilaments (16).

Despite such studies strongly suggesting that transmembrane interactions involving cytoskeletal structures do occur, further progress has been hampered by failure to identify the surface components that directly mediate cell-substratum adhesion. To this end, we have constructed a polystyrene surface to which lactoperoxidase (LPO) is covalently coupled and used it to radiiodinate the surface proteins of attached intact cells (4). Preliminary results indicated that the major protein labeled in L cells attached to this substratum was similar to a 55,000 mol wt component of the cytoskeleton (10). The present study extends these findings to another murine cell system and indicates that an identical surface protein labeled by soluble LPO is in continuity with the cytoskeleton.

MATERIALS AND METHODS

L929 cells were maintained as previously described (4). Murine peritoneal macrophages were obtained from DBA mice 4 days after intraperitoneal injection of thioglycolate broth (Scott Laboratories, Inc., Fiskeville, R. I.). Both types of cells were washed in phosphate-buffered saline (PBS) and plated onto the appropriate surface at a density of $2 \times 10^7/\text{cm}^2$ in Dulbecco’s modification of Eagle’s essential medium without serum. Incubation was carried out for 3 h at 37°C.

Lactoperoxidase-catalyzed iodination

Polystyrene surfaces to which LPO was coupled (LPO-DMS-polystyrene) were prepared as described previously (3). Cells attached to ordinary tissue culture flasks or to LPO-DMS-polystyrene were labeled with or without added LPO, respectively. After labeling, monolayer cells were washed four times with PBS, scraped off with a rubber policeman, centrifuged, and dissolved in 1% SDS. Any material remaining on the flask surface was removed with 0.2% SDS. All material was heated at 100°C for 1 min after addition of 2-mercaptoethanol to a final concentration of 1%. The substratum-bound material was concentrated fivefold by lyophilization. Aliquots were taken for analytical and preparative electropho-
resis in 5-15% linear gradient SDS polyacrylamide slab gels. Stained and dried gels were autoradiographed to determine the distribution of labeled peptides.

Detergent-extracted cytoskeletons were prepared by the method of Hynes and Destree (9) and iodinated as previously described (4). Cytoskeletons were prepared from previously iodinated intact monolayer cells in exactly the same manner.

Peptide Mapping by Limited Proteolysis and SDS Polyacrylamide Gel Electrophoresis

The procedure is a modification of the one described by Cleveland et al. (5) performed as previously described (4) except that 0.5 μg of *Staphylococcus aureus* V8 protease was used per well. To better resolve the bands in the 50,000-55,000 mol wt region, these sections of the preparative gels were excised and a Mickle gel slicer (Mickle Laboratory Engineering Co., Gomshall, Surrey, England) was used to cut them into a series of 1-mm-thick slices aligned perpendicular to the long axis of the lane. Slices containing the bands of interest were identified by determining their radioactivity in a Packard Autogamma Scintillation Spectrometer (Packard Instrument Co., Downers Grove, Ill.). One 10-slot mapping gel was used to analyze the slices from each lane of the preparative gels.

RESULTS

Electrophoretic Patterns of Iodinated Polypeptides from L929 Cells and Activated Macrophages

When soluble LPO was used to label attached L929 cells, the electrophoretic pattern of iodinated polypeptides (Fig. 1 a) was essentially the same as that previously described (4). Although there was very little labeling in the 257,000 mol wt region corresponding to the position of fibronectin, there were prominent bands at 135,000-150,000, 50,000-55,000, 42,000, and 30,000 mol wt. A diffuse band at 90,000 mol wt co-migrated with LPO. Most of the labeled material remained cell-associated after scraping. When the immobilized LPO was used to label L929 cells, the band pattern was qualitatively similar to that obtained with the soluble enzyme. However, both the distribution of radioactivity between cell- and substratum-bound material and the relative intensities of the various bands were markedly different. In this case, most of the labeled materials remained substratum-bound after scraping (Fig. 1 b). Whereas only 1.6% of the radioactivity incorporated by soluble LPO was found in the peaks at 50,000-55,000 mol wt, 5.1% of the label incorporated by the immobilized enzyme was located in this region. Because the immobilized enzyme incorporated ~4% as much label as the soluble enzyme, the increased prominence of the 50,000-55,000 mol wt bands has resulted from relatively decreased labeling of the bands outside this region.

The overall labeling pattern of macrophages by soluble LPO (Fig. 1 c) is rather similar to that of the L cells as was the tendency of the labeled material to remain cell-associated after scraping. Again, immobilized LPO labeled bands of 50,000-55,000 mol wt more intensely than any of the other bands (Fig. 1 d). It is now possible to see two bands of nearly equal intensity rather than predominance of the 55,000 mol wt band as in the L929 cells. Whereas 1.9% of the radioactivity incorporated by soluble LPO was in these two bands, they contained 13% of the label incorporated by immunobilized LPO. Because the immobilized LPO incorporated only 12% as much label as the soluble enzyme, the increased prominence of these bands has again resulted from relatively decreased labeling of bands outside of the 50,000-55,000 mol wt region.

When iodinated intact cells were extracted with the nonionic detergent, thereby removing most of the soluble proteins, nearly all of the labeled bands remained associated with the nonextractable residue (compare Fig. 2, lanes d and e). Iodination of unlabeled cytoskeletal material followed by extensive washing yielded the patterns of stained and labeled bands shown in Fig. 2, lanes c and f, respectively. The labeling pattern is somewhat different from that obtained when intact cells are labeled in that the 50,000-dalton band is not labeled and a 135,000-dalton band replaces that of 150,000 daltons.

Peptide Mapping by Limited Proteolysis and SDS Polyacrylamide Gel Electrophoresis

When the 50,000-55,000 mol wt regions from preparative gels of substratum-bound material obtained after labeling of

![Figure 1](image1.png)  
**Figure 1** SDS polyacrylamide gel analysis of L929 cells and activated macrophages iodinated by soluble or immobilized LPO. Autoradiograms of (a and c) L929 cells or macrophages, respectively, attached to polystyrene and labeled with soluble LPO. (b and d) Substratum-bound material from L929 cells or macrophages, respectively, labeled after attachment to LPO-DMS-polystyrene.

![Figure 2](image2.png)  
**Figure 2** SDS polyacrylamide gel analysis of iodinated cytoskeletal preparations from L929 cells. Coomassie Blue-stained patterns of (a) iodinated intact cells; (b) iodinated cells after detergent extraction, and (c) detergent-extracted cytoskeletons after iodination and washing. (d-f) corresponding autoradiograms.
L929 cells or activated macrophages with immobilized LPO were sliced as described, the labeled bands were easily identified and were always separated by two to three slices (corresponding to their 2- to 3-mm separation in the preparative gels). The series of peptide maps obtained from L929 and activated macrophage substratum-bound material are shown in Fig. 3A and B, respectively. In both figures, lanes 1 and 2 are derived from the 55,000 mol wt peak, while lanes 5 and 6 are derived from the 50,000 mol wt peak. The patterns of iodinated fragments in lanes 1 and 2 are very similar, as expected from the fact that the slices were derived from the same band. Because the maps from the 55,000 mol wt region are also identical for L929 cells and activated macrophages, it appears that immobilized LPO labels the same polypeptide in both cell types. It is quite striking that the pattern seen in lane 2 is repeated in lane 5 which is derived from the 50,000 mol wt band. The labeling intensities of fragments from the two mol wt regions are approximately equal in the macrophage gel, whereas fragments from the 50,000 mol wt region are considerably less intense in the L929 gel. These relationships are as expected from the relative intensities of the bands in Fig. 1. Similar results were obtained when intact cells were labeled with soluble LPO and the 50,000–55,000 mol wt regions mapped (data not shown).

Iodinated detergent-extracted cytoskeletons were mapped as described above to determine whether they contained components corresponding to the major bands labeled by immobilized LPO. Comparing Fig. 3C (lane 2), derived from the major 55,000 mol wt cytoskeleton polypeptide, with the corresponding lanes of Fig. 3A and B, it can be seen that the patterns are the same except for minor differences in resolution of the lower molecular weight fragments. The pattern in Fig. 3C (lane 2) is not repeated in the 50,000 mol wt region of Fig. 3C, suggesting that the latter band is not present in cytoskeletons. At this point, it appears to be established that the 55,000 mol wt band labeled by immobilized LPO has an identical counterpart in the detergent-extracted cytoskeletons.

When cytoskeletons were prepared from previously iodinated intact L929 cells and the 50,000–55,000 mol wt regions were mapped, it could be seen (Fig. 3D) that an iodinated component remained associated with the 55,000 mol wt band and gave rise to a pattern identical to that obtained either by iodinating cytoskeleton itself with soluble LPO or by labeling attached cells with the immobilized enzyme.

**DISCUSSION**

The data presented above contribute to two major conclusions. First, the 55,000 and 50,000 mol wt polypeptides preferentially labeled by immobilized LPO are related to one another to the extent that their peptide maps are the same even though their molecular weights differ. Presumably, more detailed mapping experiments using different proteases at a variety of concentrations would reveal the differences that must exist.

Second, these polypeptides are identical to cell surface components that remain associated with the cytoskeleton after detergent extraction. This latter finding is supported by other work (1) indicating that essentially all LPO-labeled surface components remain associated with the cytoskeleton after detergent extraction. The finding is integral to the present work because it strongly suggests that the polypeptides preferentially labeled by immobilized LPO preexist on the cell surface before attachment and are not merely leaked out of dead cells. Although 10-nm filaments were not purified by reassembly (19), a great deal of data from other laboratories indicates that the detergent extraction procedure used in these experiments removes all major cytoplasmic proteins except those in intermediate filaments and microfilaments, leaving no doubt that intermediate filaments are the source of the 55,000 mol wt polypeptide (2, 9, 17). Thus, the present study supports the conclusion that a component of 10-nm filaments is associated with the plasma membrane and comes into close approximation with the substratum during cell attachment.

Although the peptide mapping data point to a transmembrane interaction between intracellular filaments and the substratum at sites of attachment, it should not be inferred that 10-nm filament subunits are transmembrane proteins in the standard sense. If they were, then one would expect partial proteolysis to yield different sets of iodinated peptides, depending on whether subunits were labeled by immobilized LPO or whether cytoskeleton was labeled by soluble LPO. Although such a finding has been made in the case of a 100,000 mol wt transmembrane protein (6), we did not observe this result in our system. Therefore, we are inclined to think of 10-nm filaments per se, rather than their isolated subunits, as being transmembrane structures at attachment sites.

The data on activated macrophages are particularly useful because the relatively intense labeling of the 50,000 mol wt band provides evidence that the much more faintly labeled band of the same molecular weight in the L929 substratum-bound material is a real entity. Although this polypeptide exists on the macrophage surface and comes into close approximation with the substratum, we have not yet been able to determine whether it is present in or continuous with the cytoskeleton. Considering the high extracellular proteolytic activity of mac-

![Figure 3](image-url)
rophanages, it may be a partially proteolyzed, although still functional, derivative of the 55,000 mol wt polypeptide. The observation that purified intermediate filament preparations are quite susceptible to proteolysis yielding a variety of lower molecular weight fragments (17, 19) provides some precedent for this interpretation.

Other investigators have suggested that a 195,000 mol wt, trypsin-resistant polypeptide that is not large, external transformation-sensitive protein or fibronectin plays a critical role in macrophage adhesion (14). Although our labeling protocols were similar, no polypeptide of this molecular weight was iodinated by either soluble or immobilized LPO. It remains for future experiments to resolve this discrepancy and to determine whether the unique properties of macrophage attachment can be explained in terms of our data.

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