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Identification of transmembrane bridging proteins in the plasma membrane of cultured mouse L cells

(transglutaminase labeling/phagosome/immunoadsorbent affinity chromatography)

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ABSTRACT **Studies were carried out to identify transmembrane bridging proteins in the plasma membrane of mouse L-929 cells. Cells grown in suspension culture were ^{125}I-labeled by lactoperoxidase and allowed to ingest latex particles to produce inside-out membrane phagosome preparations. Phagosomes were isolated and the inner membrane surface was labeled with *N*-(5′-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide (dansylcadavarine) by a transglutaminase-catalyzed reaction. The phagosome membrane proteins were solubilized and dansylcadavarine-labeled proteins were isolated by anti-dansyl immunoadsorbent affinity chromatography. Dansylcadavarine-labeled proteins were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography for the presence of ^{125}I-labeled material. By this technique, two iodinated proteins with molecular weights of approximately 50,000 and 80,000 appear to be selectively retained by the anti-dansyl immunoadsorbent, suggesting that these proteins span the plasma membrane.**

In eukaryotic cells the plasma membrane appears to mediate the recognition of and cellular response to a wide variety of external factors. Little is known of the actual mechanisms or the identities of the proteins that are presumably of primary importance in what has been termed transmembrane control. One concept is that there are transmembrane bridging proteins that extend from the exterior of the cell through the lipid bilayer and that may interact with other proteins at the internal surface.

Direct evidence of transmembrane bridging proteins has been convincingly demonstrated in erythrocyte plasma membrane (1, 2). In addition to substantial indirect evidence that such proteins may exist in the plasma membrane of nucleated cells (3), a high molecular weight protein of mouse L cells (4) and a component of kidney (Na^+-K^+)ATPase (5) have been reported to be transmembrane bridging. However, identification and study of other transmembrane bridging proteins has been limited in part by the fact that techniques useful in studies on erythrocytes have proven more difficult to apply to membrane from other sources. In the present study, mouse L cell phagosome preparations were used as a source of inside-out plasma membrane (4, 6). In this paper we report the use of L cell phagosomes and a double labeling technique to identify proteins that are potentially transmembrane bridging.

MATERIALS AND METHODS

Cell Culture. Mouse L-929 cells were grown in suspension culture in minimal essential medium supplemented with 5% calf serum, 0.15% methylcellulose (15 CPS), 40 units of penicillin per ml, and 40 μg of streptomycin per ml at 37°. Cells were suspended in fresh medium at a density of 3×10^4 cells per ml approximately 24 hr before iodination.

Iodination. Cells were rinsed three times in minimal essential medium and resuspended in phosphate-buffered saline minus Ca^{2+} and Mg^{2+} and containing 5 mM glucose, and lactoperoxidase-catalyzed iodinations were performed by a modification of the method described by Hynes (7). The reaction medium contained approximately 5×10^7 L cells, 5 mM glucose, 100 μg of lactoperoxidase (67 units/mg), 10 μg of glucose oxidase (277 units/mg), 40 nmol of NaI, and 5 mCi of $Na^{125}I$ (carrier free) in 4 ml of phosphate-buffered saline. After a 10-min labeling period the cells were rinsed in phosphate-buffered saline containing 1 mM tyrosine, then three times in minimal essential medium.

Latex-Filled Phagosome Formation. ^{125}I-Labeled cells were resuspended to 10^6 cells per ml in minimal essential medium supplemented with 5% calf serum containing 10^4 latex beads (Polyscience, 1.01 μm dia) per cell. The latex beads and cells were incubated for 60 min at 37° on a rotatory shaker and latex-filled phagosomes were isolated by the method of Wetzel and Korn (8) as adapted for L cells (4, 6). The recovered latex–membrane vesicles were diluted with an equal volume of 125 mM KCl/20 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)/2 mM $CaCl_2$ at pH 7.2 and centrifuged at $15,000 \times g$ for 20 min. The resulting pellet was then gently resuspended in KCl/Hepes buffer.

Dansylcadavarine Labeling of Latex-Filled Phagosomes. Transglutaminase (molecular weight 85,000) was isolated from guinea pig liver (9) to better than 95% purity as estimated by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. Conditions for transglutaminase-catalyzed labeling of membrane vesicles were similar to those previously described for labeling of erythrocyte ghosts (10, 11). The reaction mixture contained 125 mM KCl, 20 mM Hepes, 5 mM dansylcadavarine [*N*-(5′-aminopentyl)-5-dimethylamino-1-napthalenesulfonamide], 2 mM $CaCl_2$, and 10 μM transglutaminase at pH 7.5. The labeling reaction was conducted at 37° for 60 min. The solution was then cooled to 4° and centrifuged at $15,000 \times g$ for 20 min, and the resulting pellet was resuspended in a solution containing freshly prepared 60 mM borate/1% Triton X-100/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride/2 mM *N*-ethylmaleimide/2 mM iodoacetamide/2 mM dithiothreitol at pH 7.8. The solution was agitated and centrifuged again at $15,000 \times g$ for 20 min. The supernatant was then applied to a 1.5×40 cm Bio-Rad P-10 column and eluted with borate/Triton X-100 buffer containing 1 mM EDTA. The excluded protein fraction was collected for affinity chromatography.

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; dansylcadavarine, *N*-(5′-aminopentyl)-5-dimethylamino-1-naphthalenesulfonamide.

Anti-Dansyl Affinity Chromatography. Rabbit IgG purified by Sepharose-protein A affinity chromatography was dansylated by the method of Gray (12). Rabbits were given weekly subcutaneous injections of 0.5 mg of dansylated IgG in Freund's complete adjuvant for 5 weeks. Seven days after the final injection, the rabbits were exsanguinated and a serum fraction was recovered. An IgG-containing ammonium sulfate fraction was prepared, dialyzed, and immobilized on CNBr-activated Sepharose 4B. Columns (0.9 × 10 cm) of anti-dansyl immune serum-Sepharose 4B were prepared and equilibrated with 60 mM borate/1% Triton X-100 at pH 7.8. Similar columns with nonimmune rabbit IgG-Sepharose 4B were also prepared. Anti-dansyl affinity columns prepared in this manner would retain dansylated albumin but not albumin alone.

Samples of dansylcadavarine-labeled protein solubilized from the ^{125}I-labeled phagosome preparation were applied in a total volume of 5 ml to tandem nonimmune, anti-dansyl affinity columns at room temperature. The columns were arranged so that the sample passed first through the nonimmune IgG column then through the anti-dansyl column. The columns were then washed with borate/Triton X-100 buffer containing 1 M KCl until no ^{125}I radioactivity could be detected in the effluent. The columns were then separated and retained material was eluted with 50 mM citrate/1% Triton X-100 at pH 2.8. Samples of phagosome preparation and proteins from the retained column fractions were precipitated in acidified acetone (13), washed with ethanol/ether (1:1 vol/vol), dried under nitrogen, and resuspended in a small amount of buffer for $NaDodSO_4$/polyacrylamide gel electrophoresis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Samples were diluted in an equal volume of 0.14 M Tris/22.3% glycerol/0.12 M 2-mercaptoethanol/6% $NaDodSO_4$/0.001% bromphenol blue at pH 6.8. The samples were boiled for 3 min and electrophoresis was performed by a modification of the method of Laemmli (14) as described by Studier (15). After electrophoresis, the gels were photographed under UV light. The gels were then fixed and stained in 50% trichloroacetic acid/0.1% Coomassie blue at 37° for 2 hr and destained in 10% acetic acid/5% methanol. Gels were dried onto filter paper for autoradiography.

RESULTS

Phagosome Preparations. Evidence has been presented that phagosomes prepared from L cells possess plasma membrane that is primarily "inside-out" (4, 6). Since this assumption is of critical importance, it was necessary to determine that the phagosomes produced for the present study had the same characteristics. Cells and phagosomes were incubated in phosphate-buffered saline containing 10 μg of trypsin per ml for 10 min at 37°. The preparations were rinsed twice in 10 volumes of phosphate-buffered saline and acid-precipitable ^{125}I was determined. Approximately 90% of acid-precipitable ^{125}I incorporated into intact L cells by lactoperoxidase could be solubilized by treatment with trypsin, while less than 5% of the ^{125}I was solubilized from phagosomes prepared from iodinated L cells under the same conditions. In incubations without trypsin, less than 5% of the total acid-precipitable ^{125}I could be recovered in a soluble fraction from intact cells and less than 2% of the total was released from phagosomes prepared from these cells. These results are similar to the observations of Hunt and Brown (4).

Transglutaminase-Catalyzed Incorporation of [^{14}C]-Cadavarine. Transglutaminase, an endo-γ-glutamine:ε-lysine transferase, catalyzes the covalent incorporation of a number of synthetic derivatives of primary amines into proteins, via an acyl transfer of γ-amide of glutamine residues in proteins (11). This technique has been used with the fluorescent amine derivative dansylcadavarine to label proteins in erythrocyte ghosts (10, 11), sarcoplasmic reticulum (10), and cultured fibroblasts (16). Initial experiments indicated that [^{14}C]cadavarine was poorly incorporated into intact L cell preparations in the presence of transglutaminase. Incubations in the absence of enzyme resulted in a rate of incorporation approximately 5% of that observed in the presence of trans- glutaminase. However, as shown in Fig. 1, under similar conditions, the rate of transglutaminase-catalyzed incorporation of [^{14}C]cadavarine into phagosomes from a given number of cells was nearly 4 times greater than the rate of incorporation into intact cells. Endogenous incorporation of cadavarine under these conditions was negligible. Under the conditions used in this study approximately 10% of ^{125}I incorporated into intact L cells after lactoperoxidase iodination is recovered in the phagosome preparation. If it is assumed that this represents roughly 10% of the plasma membrane from a given number of cells recovered in phagosome preparations, then the differences in the rate of transglutaminase-catalyzed incorporation of [^{14}C]cadavarine between intact cells and phagosomes are approximately 10 times greater when expressed on an equivalent membrane basis.

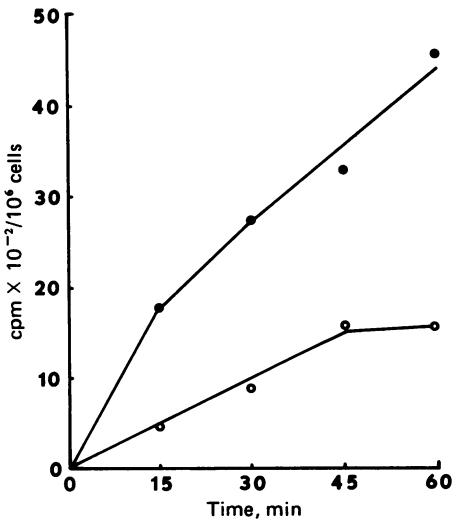

FIG. 1. Transglutaminase-catalyzed incorporation of [^{14}C]-cadavarine into (○) L cells and (●) phagosomes. Cells (2×10^6) were incubated in 0.5 ml of phosphate-buffered saline containing 2 mM cadavarine (1 μCi of [^{14}C]cadavarine), 1 μM transglutaminase, and 1 mM $CaCl_2$. At the indicated times the incubations were centrifuged and resuspended three times in phosphate-buffered saline and the acid-precipitable radioactivity was determined. Incubations were carried out in the presence and absence of enzyme; values represent the mean of duplicate determinations minus endogenous incorporation.

Identification of Transmembrane Bridging Proteins in Phagosomal Preparations. The experimental protocol is summarized in Fig. 2. Proteins detected by this technique, which are ^{125}I-labeled and retained by the anti-dansyl column, are inferred to have sequences available at both inner and external membrane surfaces and are assumed to be transmembrane bridging.

The results of a typical experiment are given in Fig. 3. Incubation with only transglutaminase and dansylcadavarine revealed that transglutaminase (molecular weight 85,000) labels itself and can be localized in polyacrylamide gels by analysis of the fluorescence pattern. A significant amount of dansyl-

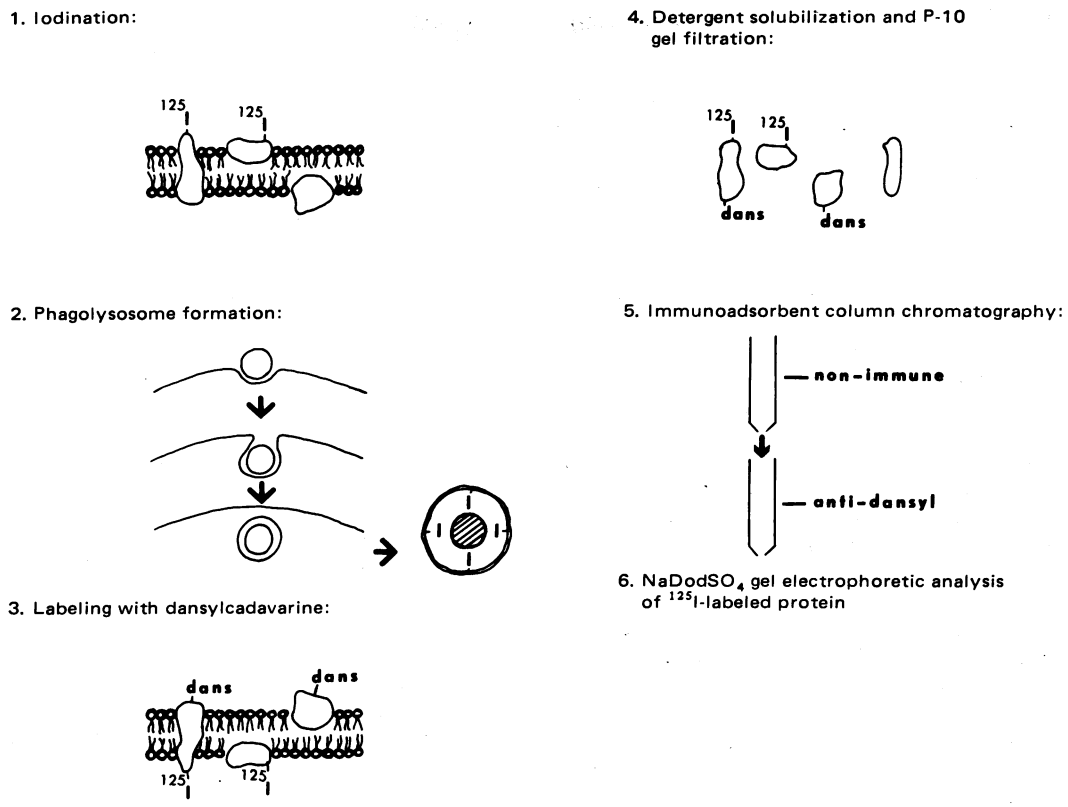

FIG. 2. Experimental protocol for identification of transmembrane bridging proteins.

cadavarine-labeled transglutaminase remained in the phagosome preparation applied to the immunoadsorbent columns and was retained by the anti-dansyl column. A number of additional bands of fluorescent material can be seen in the phagosome preparation and, within the limits of detection, many of these bands appear to have been selectively retained on the anti-dansyl column. No detectable ^{125}I or fluorescence could be visualized in samples eluted from the nonimmune column (data not shown). Comparison of the fluorescence patterns with the ^{125}I autoradiography indicates that while a number of fluorescent bands were retained by the anti-dansyl column, only three major bands containing significant ^{125}I were detected. The ^{125}I-labeled protein bands selectively retained by the anti-dansyl immunoadsorbent correspond to molecular weights of approximately 50,000, 80,000, and 95,000. The 80,000 molecular weight ^{125}I-labeled protein migrates in front of dansylcadavarine-labeled guinea pig liver transglutaminase and corresponds to an iodinated protein in phagosome preparations which is distorted by the presence of nonlabeled transglutaminase.

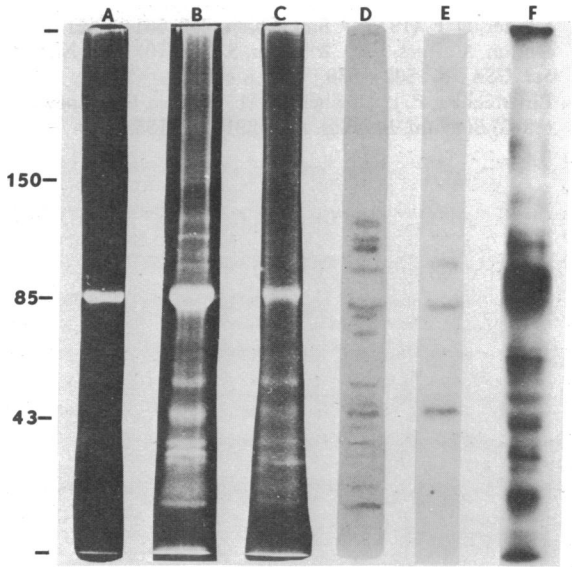

FIG. 3. $NaDodSO_4$ (5–15%)/polyacrylamide gradient gel patterns of dansylcadavarine and ^{125}I-labeled L cell proteins. Molecular weights ($\times 10^{-3}$) are shown on the left. A, Fluorescence pattern of an incubation containing only transglutaminase and dansylcadavarine. B, Fluorescence pattern of phagosome preparation applied to immunoadsorbent columns. C, Fluorescence pattern of material retained by the anti-dansyl immunoadsorbent column. D, Iodination pattern of phagosome preparation B. E, Iodination pattern of material retained by anti-dansyl immunoadsorbent column (C). F, Iodination pattern of intact L cells.

DISCUSSION

One of the major difficulties in studies on plasma membrane protein symmetry has been the difficulty in obtaining relatively pure plasma membrane with a particular orientation or "sidedness." The use of latex-filled phagosomes and lactoperoxidase-catalyzed iodination to identify surface proteins affords a unique model for such studies. The additional application of enzyme-catalyzed hapten affinity labeling has allowed the recovery of labeled protein, making possible the identification of specific molecules. Proteins detected by the technique used in the present study must meet at least three criteria. Such proteins must possess a tyrosine residue at the external surface available to lactoperoxidase and a glutamine other than an NH_2-terminal residue at the cytoplasmic surface available to transglutaminase and must be included in the phagosome membrane after endocytosis of the latex particle. It is therefore likely that other transmembrane bridging proteins exist and are not detected by this procedure.

Previous reports have indicated that transglutaminase labels relatively few proteins in erythrocyte membrane (10, 11) and sarcoplasmic reticulum (10, 17). In the present study, the observed difference in the rate of transglutaminase labeling between intact cells and phagosomes indicated that the cytoplasmic surface is labeled more efficiently than the external plasma membrane surface. The reason for this apparent difference in the availability of glutamine is not clear. It may simply reflect differences in the amount of protein present or an asymmetric distribution of available glutamine residues. The situation becomes more complex when it is considered that L cell plasma membrane proteins have been reported to contain γ-glutamyl-ϵ-lysine crosslinks (18). The presence of such isopeptide bonds could make glutamine-containing proteins unavailable to exogenous transglutaminase labeling.

The results of this study suggest that there are at least two plasma membrane proteins present in L cells which span the membrane. Estimations of molecular weight by $NaDodSO_4$/polyacrylamide gel electrophoresis indicate that these proteins are approximately 50,000 and 80,000. Although both proteins seem to coelectrophorese with major iodinatable bands from intact L cell preparations, the relative amounts of these proteins have not been determined. A third protein with an estimated molecular weight of 95,000 was also detected as transmembrane bridging. However, because transglutaminase is capable of forming γ-glutamyl-ϵ-lysine bonds and can crosslink proteins (10, 17), it must be considered that this 95,000 molecular weight protein may represent a dimer of the 50,000 molecular weight protein. Although this possibility cannot be discounted on the basis of the data presented, it seems unlikely. A preliminary study on the effect of transglutaminase on phagosome preparations failed to detect the creation of new molecular weight protein species capable of entering a 4.2% acrylamide gel after treatment of phagosome preparations with the enzyme (R. M. Evans and L. M. Fink, unpublished observations).

Using phagosome preparations from iodinated L cells, Hunt and Brown (4) demonstrated trypsin sensitivity of an iodinatable 250,000 molecular weight protein and suggested that this major surface protein spanned the membrane. L-929 cells used in these studies appear to lack a major iodinatable 220,000–250,000 molecular weight protein. Therefore, the inability of the present technique to detect this high molecular weight protein probably reflects surface differences in the cells used in the respective studies.

Further experiments, using other techniques, are clearly necessary to confirm that the proteins described in this study actually span the plasma membrane. The identification of transmembrane bridging proteins is a first step in the study of specific membrane proteins that may play a significant role in transmembrane control.

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