A 39-kD Plasma Membrane Protein (IP39) Is an Anchor for the Unusual Membrane Skeleton of *Euglena gracilis*

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Abstract. The major integral plasma membrane protein (IP39) of *Euglena gracilis* was radiolabeled, peptide mapped, and dissected with proteases to identify cytoplasmic domains that bind and anchor proteins of the cell surface. When plasma membranes were radioiodinated and extracted with octyl glucoside, 98% of the extracted label was found in IP39 or the 68- and 110-kD oligomers of IP39. The octyl glucoside extracts were incubated with unlabeled cell surface proteins immobilized on nitrocellulose (overlays). Radiolabel from the membrane extract bound one (80 kD) of the two (80 and 86 kD) major membrane skeletal protein bands. Resolubilization of the bound label yielded a radiolabeled polypeptide identical in M_r to IP39. Intact plasma membranes were also digested with papain before or after radioiodination, thereby producing a cytoplasmically truncated IP39. The octyl glucoside extract of truncated IP39 no longer bound to the 80-kD membrane skeletal protein in the nitrocellulose overlays. EM of intact or trypsin digested plasma membranes incubated with membrane skeletal proteins under stringent conditions similar to those used in the nitrocellulose overlays revealed a partially reformed membrane skeletal layer. Little evidence of a membrane skeletal layer was found, however, when plasma membranes were predigested with papain before reassociation. A candidate 80-kD binding domain of IP39 has been tentatively identified as a peptide fragment that was present after trypsin digestion of plasma membranes, but was absent after papain digestion in two-dimensional peptide maps of IP39. Together, these data suggest that the unique peripheral membrane skeleton of *Euglena* binds to the plasma membrane through noncovalent interactions between the major 80-kD membrane skeletal protein and a small, papain sensitive cytoplasmic domain of IP39. Other (62, 51, and 25 kD) quantitatively minor peripheral proteins also interact with IP39 on the nitrocellulose overlays, and the possible significance of this binding is discussed.

A membrane skeleton underlying the plasma membrane plays a key role in maintaining cell form and elasticity in at least two widely divergent cell types: mammalian erythrocytes (4, 26) and unicellular *Euglenas* (13, 14). Yet both the composition and arrangement of the major proteins of these membrane skeletons differ significantly. In erythrocytes, for example, the spectrins (~220 kD), cross-linked with nonspectrin proteins, constitute a submembrane meshwork parallel to the cell surface. In euglenoids, on the other hand, two smaller proteins (80 and 86 kD) associate stoichiometrically to generate filaments that are arranged perpendicularly to the cell surface. In erythrocytes and probably euglenas (see below) the peripheral membrane skeleton is attached to the plasma membranes through one or more integral membrane proteins. These membrane anchors are of considerable interest because they could, in theory, select the specific membrane skeletal proteins that bind to the plasma membrane and, if so, they could then determine in what regions of the cell the membrane skeleton assembles.

From the data available it appears that membrane anchors vary considerably in molecular mass and glycosylation among different cell types (2, 5, 7, 9, 11, 17, 19, 21, 28, 36, 37, 39), reflecting perhaps the requirements of individual cells for their own unique skeletal protein arrangement. In *Euglena*, a 39-kD protein (IP39),[1] apparently unglycosylated, has been identified as a possible binding site for the peripheral membrane proteins that constitute the membrane skeleton (15). IP39 readily forms dimers of 68 kD and higher order oligomers, even under the strong denaturing conditions used in SDS polyacrylamide gels. How the solution properties of IP39 might relate to some of the striking characteristics of the *Euglena* plasma membrane is unknown. Freeze-fracture experiments, for example, have revealed that the interior of the plasma membrane consists of regions of highly ordered particles or striations that alternate with less ordered areas (25). These ordered intramembrane regions coincide with the sites at which the membrane skeleton attaches to the cytoplasmic side of the membrane.

Because IP39 is the most abundant integral plasma mem-

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1. *Abbreviation used in this paper*: IP39, 39-kD integral membrane protein in *Euglena*.

brane protein and has an appropriate cytoplasmic domain, it seemed reasonable to assume that IP39 might be associated with membrane skeletal protein binding. In this paper, experiments are described that demonstrate IP39 was able to interact with a major component of the membrane skeleton. In nitrocellulose overlays, a radiolabeled IP39 bound to an 80-kD membrane skeletal protein, but not to its equally abundant 86-kD partner. Additional experiments show that only a small portion of the substantial cytoplasmic domain of IP39 was required for membrane skeletal protein binding. Binding could be demonstrated, for example, when the cytoplasmic domain of IP39 was truncated with trypsin, but not after more extensive digestion with papain. Using isolated membranes, it has also been found that membrane skeletal proteins will bind only to the plasma membrane and not to the contiguous flagellar membrane that lacks IP39. These results suggest that IP39 may play an important role in the binding and distribution of the peripheral proteins that appear to be major determinants of cell form in these cells.

Materials and Methods

Cell Surface Isolation, Fractionation, and Digestion

Cell surface isolates consisting of the plasma membrane, the underlying membrane skeleton, microtubules and bridgework, were purified from *Euglena gracilis* strain Z on sucrose gradients as previously described (13). Soluble fractions enriched in different membrane skeletal proteins were obtained by separate or sequential extraction of surface isolates for 30 min at 4°C with (*a*) urea: 4 M urea in 10 mM Tris-Cl at pH 7.5, 5 mM EDTA or (*b*) base: 20 mM NaOH in distilled water. Extraction of surface isolates with base yielded soluble peripheral proteins and insoluble "stripped" plasma membranes (13, 15). Insoluble fractions were sedimented at 12,800 *g* for 10 min. Plasma membranes were proteolyzed with (*a*) 0.5% trypsin-TPCK (13,000 BAEE U/mg solid; Sigma Chemical Co., St. Louis, MO) in 125 mM borate buffer at pH 8.4, for 1 h at room temperature, followed by washing with 0.5% soybean trypsin inhibitor (Sigma Chemical Co.) and 0.4 mM PMSF (Sigma Chemical Co.) in borate buffer, or, (*b*) 0.5% papain (12 U/mg solid, Sigma Chemical Co.) in 50 mM Hepes at pH 6.2 with 0.1 mM L-cysteine for 1 h at room temperature, followed by washing with 0.1% antipain and 25 g/ml leupeptin in 50 mM Hepes at pH 6.2 without L-cysteine. All digested membranes were washed twice with base. The major integral plasma membrane protein or its protease resistant fragments were solubilized from stripped plasma membranes with 2% octyl-β-D-glucopyranoside (octyl-glucoside) (Sigma Chemical Co.) in 10 mM Tris-Cl at pH 7.5 with 5 mM EDTA for 1 h at room temperature.

Flagella and Flagellar Membrane Vesicle Isolation

Flagella and flagellar membrane vesicles were prepared as described previously (15).

Gel Electrophoresis and Protein Blotting

Denaturing acrylamide gels (SDS-PAGE) were prepared as described (24), with the addition of 0.05% NP-40 to the running buffer to improve the resolution of the membrane proteins (15). Molecular masses were estimated from proteins electrophoresed in the absence of NP-40 as proteins and standards migrate anomalously under these conditions. Soluble samples were concentrated by TCA precipitation before electrophoresis (18). Proteins were transferred electrophoretically (38) to nitrocellulose (Trans-blot medium, Bio-Rad Laboratories, Richmond, CA) as previously described (15). Transfer of protein was assessed by staining with Ponceau S (Sigma Chemical Co.).

Membrane Iodination

Stripped plasma membranes (~200 μg) were resuspended in 0.1 ml of 100 mM sodium phosphate at pH 7.4, and transferred to a 1.5-ml microfuge tube containing one Iodobead (Pierce Chemical Co., Rockford, IL) and 200–300 μCi of carrier-free Na[^{125}I] (Amersham Corp., Arlington Heights, IL) in 0.1 ml of 100 mM sodium phosphate at pH 7.4 (27). After incubation on ice for 30 min with occasional mixing, unincorporated label was removed by washing the membrane pellet two times with buffer. The resulting radiolabeled membranes were solubilized by boiling in sample buffer before SDS-PAGE. Alternatively, proteins from the radioiodinated membranes were solubilized with 2% octyl-glucoside to generate a radiolabeled probe (see below). Probes were prepared immediately before use.

Two-Dimensional Tryptic Peptide Maps of IP39

The trypsin- or papain-resistant forms of IP39 were separated by SDS-PAGE, stained, excised, and radioiodinated using chloramine T as catalyst (16). To identify exposed, presumably cytoplasmic domains of IP39, undigested or either trypsin- or papain-digested plasma membranes were radioiodinated using Iodobeads (Pierce Chemical Co.) before solubilization for SDS-PAGE. After electrophoresis, stained gel slices containing these radioiodinated forms of IP39 ([^{125}I]IP39, [^{125}I]IP39$_{trypsin}$, [^{125}I]IP39$_{papain}$) were excised and dried. The labeled gel slices were then digested with trypsin and the peptide fragments separated by two-dimensional thin layer chromatography (15).

Nitrocellulose Overlay

Proteins from cell surface isolates were separated by SDS-PAGE and transferred to nitrocellulose as described above. The nitrocellulose was washed overnight in PBS and blocked for 1 h at room temperature in 10 mM sodium phosphate at pH 7.8 with 1% BSA, 150 mM NaCl, 0.1% gelatin, 0.5% NP-40, 0.1% SDS and 0.1% β-mercaptoethanol (overlay buffer). Blots were then incubated for one hour at room temperature with radioiodinated IP39 ([^{125}I]IP39) or its papain resistant derivative ([^{125}I]IP39$_{papain}$), first labeled and then extracted from the plasma membrane with 2% octyl-glucoside. The radiolabeled probes were diluted at least 100-fold with fresh overlay buffer before incubation. Blots were subsequently washed at least 10 times for 3 min each, with overlay buffer without BSA, dried and autoradiographed using Kodak XRP-1 film (Eastman Kodak Corp., Rochester, NY) and a Cronex intensifying screen (Dupont Co., Wilmington, DE). Radioiodinated proteins bound to the 80-kD protein blotted to the nitrocellulose were eluted by boiling the 80-kD region of the nitrocellulose in SDS-PAGE sample buffer (24) containing 6 M urea.

Reassociation of Membrane Skeletal Proteins with NaOH-stripped Plasma Membranes

NaOH-stripped plasma membranes or NaOH-stripped membranes digested with trypsin or papain were combined in a 10-mm dialysis tube with a two-fold (vol/vol) excess of membrane skeletal proteins extracted from cell surface isolates with 4 M urea, and dialyzed at 4°C for 1 h against overlay buffer without BSA and gelatin (see above). The insoluble fraction was sedimented and washed once on ice with 100 mM Pipes at pH 7.0 with 150 mM NaCl, 0.5% NP-40 and 0.1% SDS. Reassociation pellets were solubilized for SDS-PAGE or fixed, dehydrated, embedded, and sectioned for transmission EM as described (13). Stained sections were examined and photographed with a JEOL 1200 EX transmission electron microscope.

Results

Monomeric and Oligomeric Forms of IP39

Peripheral membrane proteins of *Euglena gracilis* were removed from cell surface isolates with base (13) and the resulting "stripped" plasma membranes lost all peripheral proteins as well as the ridge and groove form that characterizes whole cells, surface isolates and trypsin-digested surface isolates. Incubation of stripped membranes with the NaOH extract (i.e., membrane skeletal proteins) resulted in the reformation of a membrane skeletal layer (14), indicating that the plasma membrane retained appropriate membrane skeletal protein binding sites. SDS acrylamide gels (24) of the stripped plasma membranes revealed two major polypep-

tide bands with apparent M_rs of 39 and 68 kD (13). After isoelectric focusing (29) the 68 kD polypeptides were mostly converted to 39-kD monomers (IP39) with isoforms ranging from pH 5.5 to 6.5 (15). This conversion was clearly favored by the presence of some component of the isoelectric focusing sample buffer that has subsequently been identified as NP-40 (Rosiere, T. K., and G. B. Bouck, unpublished observations). Adding NP-40 to the sample and gel running buffer in SDS gels resulted in better resolution of the 39- and 68-kD bands and generally in preferential accumulation of the 39-kD band. The effects of neutral detergents on the apparent M_r of membrane proteins has been previously discussed (20).

Identification of Cytoplasmically Exposed Domains of IP39

IP39 could not be detected on the outer surface of the cell after radioiodination or immunolabeling of intact cells (15). By contrast, when the inner membrane surface was exposed, as in surface isolates or NaOH-stripped plasma membranes, IP39 was heavily radioiodinated (15). Trypsin or papain digestion of similar NaOH-stripped plasma membranes (Fig. 1, lanes *2* and *3*; reference 14) reduced the molecular mass of IP39 by ~25 (M_r = 30 kD) and 50% (M_r = 22 kD), respectively.

To distinguish protease-sensitive domains of IP39 from membrane protected and/or protease insensitive regions, plasma membranes were digested with trypsin or papain, solubilized and separated by SDS-PAGE. The IP39 band from the acrylamide gel of the trypsin and papain digested plasma membranes was excised from the gel, radioiodinated using chloramine T as the catalyst and then digested with trypsin (16). The tryptic fragments were then separated by two-dimensional thin layer chromatography (cf. schematic in Fig. 2, pathway 3, *a* and *b*). In this experiment all the radiolabeled tryptic fragments of IP39 will be revealed except those exposed to and removed by proteolysis of the intact membrane. Autoradiographs of the two-dimensional peptide maps of IP39 from trypsin-digested stripped membranes (Fig. 3 *a*) and IP39 from papain digested stripped membranes (Fig. 3 *b*) were markedly different, but in both maps a set of tryptic peptides was absent (brackets, Fig. 3, *a* and *b*) when compared with the tryptic peptide map of IP39 from undigested stripped membranes (Fig. 3 *f*; reference 15). These missing peptides were therefore presumed to be those exposed to digestion on the cytoplasmic side of the plasma membrane.

Figure 1. SDS-PAGE of plasma membranes after protease digestion. Cell surfaces extracted with 20 mM NaOH (stripped plasma membranes, lane *1*) were digested for one hour at room temperature with 0.5% trypsin (lane *2*) or 0.5% papain (lane *3*). Relative amounts of membranes: undigested (lane *1*), 1×; trypsin-digested (lane 2), 1.5×; papain-digested (lane 3), 2×. Coomassie brillian blue–stained gel.

The identity of these cytoplasmic fragments was further tested by radiolabeling only the cytoplasmically exposed regions of IP39. In these experiments the catalyst for iodination was attached to a solid matrix (Iodobeads, Pierce Chemical Co.) incapable of penetrating the lipid bilayer. Undigested, trypsin- or papain-digested NaOH stripped plasma membranes were first radioiodinated with Iodobeads, and then solubilized in sample buffer and separated by SDS-PAGE. The cytoplasmically labeled IP39 ([^{125}I]IP39) and its corresponding protease digested and radiolabeled forms ([^{125}I]IP39$_{trypsin}$, [^{125}I]IP39$_{papain}$) were excised from the denaturing acrylamide gels, digested with trypsin, and the resulting peptides separated by two-dimensional thin layer chromatography (cf. Fig. 2, pathways 3, *c*, *d*, and *e*). The radiolabeled tryptic fragments present in the peptide maps of [^{125}I]IP39 (Fig. 3 *c*), [^{125}I]IP39$_{trypsin}$ (Fig. 3 *d*) and [^{125}I]-IP39$_{papain}$ (Fig. 3 *e*) were therefore cytoplasmic. The 22-kD papain-digested form of IP39 (Fig. 1, lane *3*) still clearly contained a iodinatable cytoplasmic domain (Fig. 3 *e*).

Thus specific cytoplasmic fragments that were present in the map of [^{125}I]IP39 (brackets, Fig. 3 *c*) were absent when membranes were digested with trypsin or papain (brackets, Fig. 3, *d* and *e*). These missing peptides confirmed the results shown in Fig. 3, *a* and *b* in that membrane labeling followed by SDS-PAGE (Fig. 3, *d* and *e*) or SDS-PAGE followed by labeling of specific integral membrane proteins (Fig. 3, *a* and *b*) identified a similar set of cytoplasmic, protease sensitive, tryptic fragments of IP39.

The data from these peptide maps and from reference 15 were combined to generate a composite peptide map of IP39. The peptides most clearly resolved in these experiments are indicated as cytoplasmic (*open circles*) or membrane (*filled* and *stippled circles*) in Fig. 3 *f*. The abundance of cytoplasmic (radioiodinated with Iodobeads) tryptic fragments relative to the few clearly assignable to the membrane domain was unexpected and may indicate fewer protease sensitive sites in the membrane embedded region or fewer iodinatable sites in this region. One well defined cytoplasmic peptide fragment may be of especial interest (see below) as it is present after trypsin digestion, but absent after papain digestion (*arrows* in Fig. 3, *c*, *d*, *e*, and *f*).

Detergent-solubilized [^{125}I]IP39 Binds to Specific Membrane Skeletal Proteins

NaOH stripped plasma membranes were radiolabeled and then extracted with 2% octyl-glucoside to make soluble IP39 probes (cf. schematic in Fig. 2). As seen in Fig. 4, the only significantly labeled proteins in acrylamide gels of these extracts were [^{125}I]IP39 (and 68- and 110-kD oligomers of IP39, see reference 15). Densitometry of the complete autoradiogram of the probe shown in Fig. 4 showed that >98% of the radiolabel was associated with proteins with M_rs of 39, 68, and 110 kD. The two probes (undigested and papain

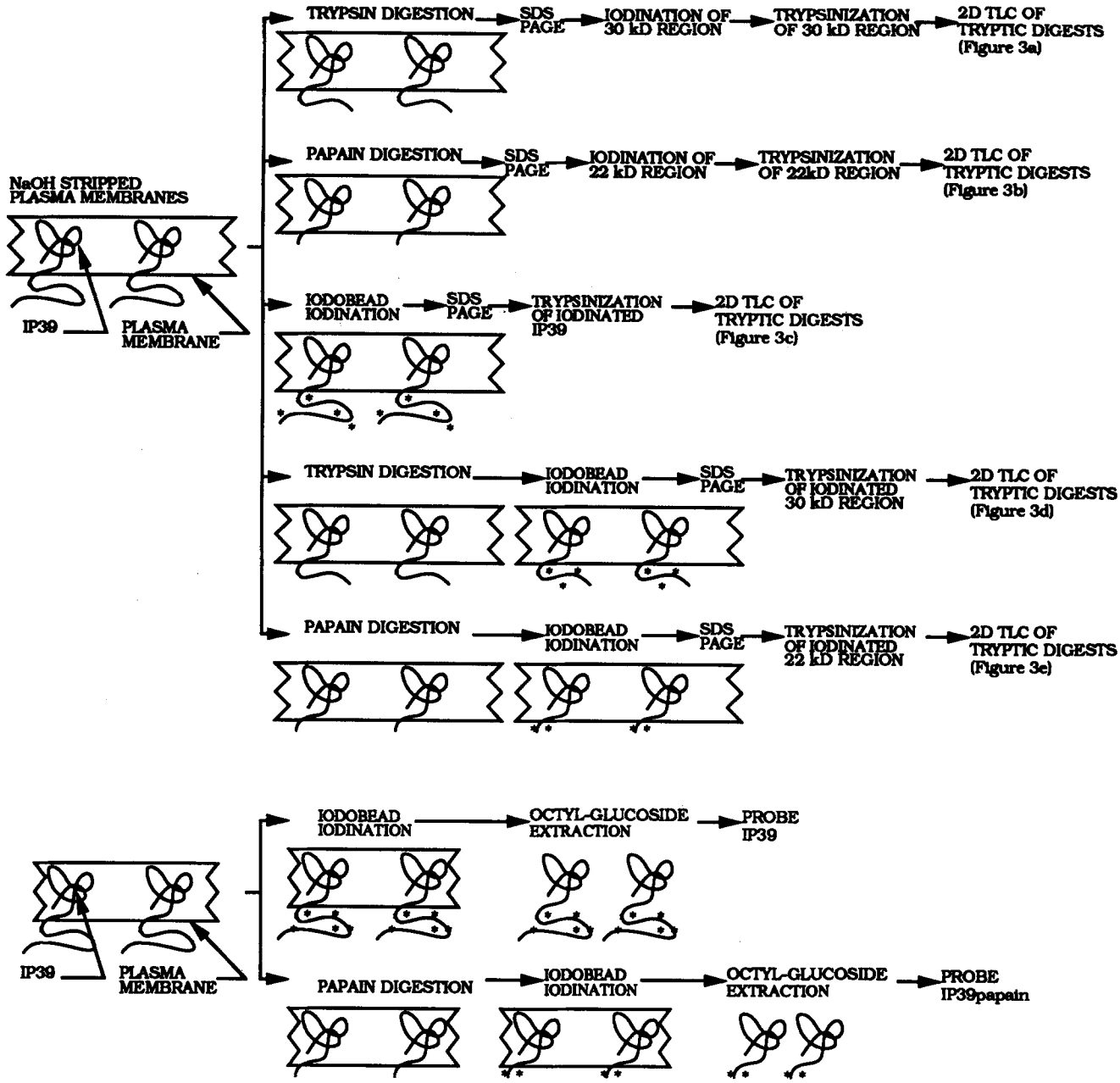
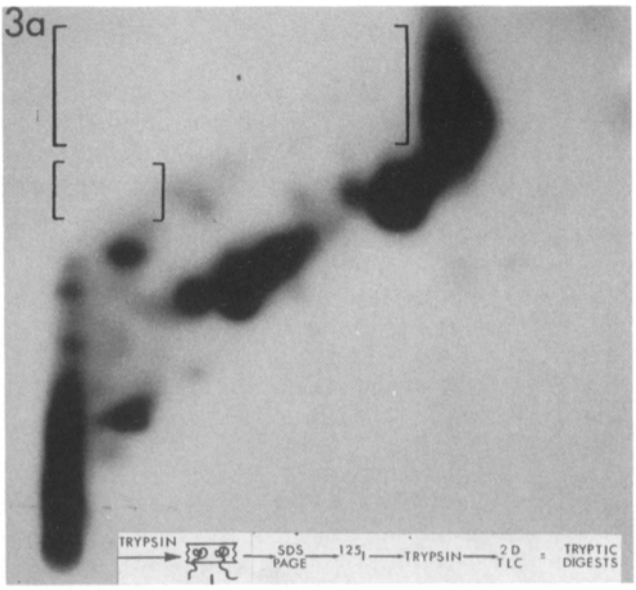


Figure 2. Schematic outlining the general method of constructing IP39 probes for nitrocellulose overlays, and for iodination of IP39 for peptide maps. In all these experiments NaOH-stripped plasma membranes were derived from surface isolates purified on sucrose gradients. Radiolabeling was carried out after separation of IP39 by SDS-PAGE (Fig. 3*a*), using chloramine T as the catalyst, or before solubilization of membranes (Fig. 3, *c–e*) using Iodobeads followed by gel separation. In both methods, the final trypsin digestion was carried out on the 39-kD band or its 22- and 32-kD derivatives in the acrylamide gel (16).

digested) were used to assess binding of IP39 to membrane skeletal proteins immobilized on nitrocellulose filters.

Various protein fractions from cell surface isolates were separated by SDS-PAGE and stained with Coomassie brilliant blue (Fig. 5 *a*). Identical fractions from the same gel were transferred to nitrocellulose and incubated with aliquots of the same [^{125}I]IP39 and [^{125}I]IP39$_{papain}$ probes shown in Fig. 4. Under stringent conditions (see Materials and

Figure 3. Two-dimensional peptide maps of IP39 after digestion of the cytoplasmic domain with trypsin (*a*) or papain (*b*). Radiolabeled peptides from both the membrane embedded and the undigested cytoplasmic regions of IP39 are revealed in these maps. In *c*, *d*, and *e* are two-dimensional peptide maps of IP39 radiolabeled with Iodobeads in intact or digested plasma membranes. The membranes were undigested (*c*), trypsin-digested (*d*), or papain-digested (*e*) before radioiodination. Only peptides from the cytoplasmic regions of IP39 are radiolabeled in these autoradiographs. In the composite tryptic map (*f*), open circles indicate cytoplasmic peptides, closed circles are well-labeled membrane peptides and stippled circles are faintly labeled membrane peptides. This diagram was constructed from the data in *a–e*, and reference 15. The broken circles are anomalous fragments that appeared in *c* but not in the peptide maps of reference 15.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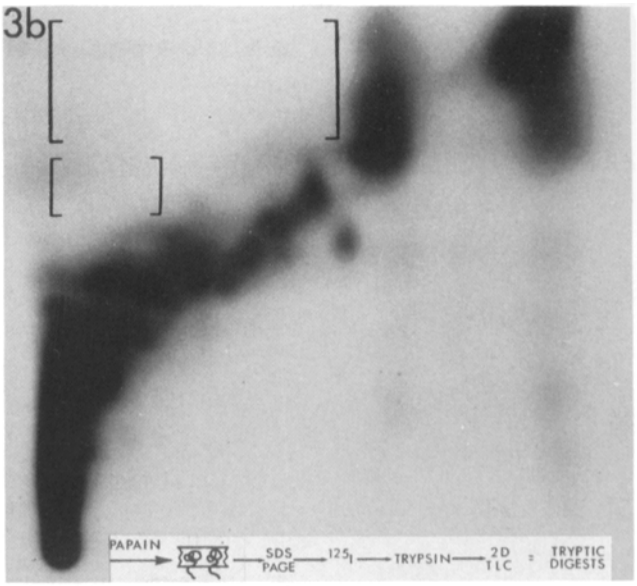
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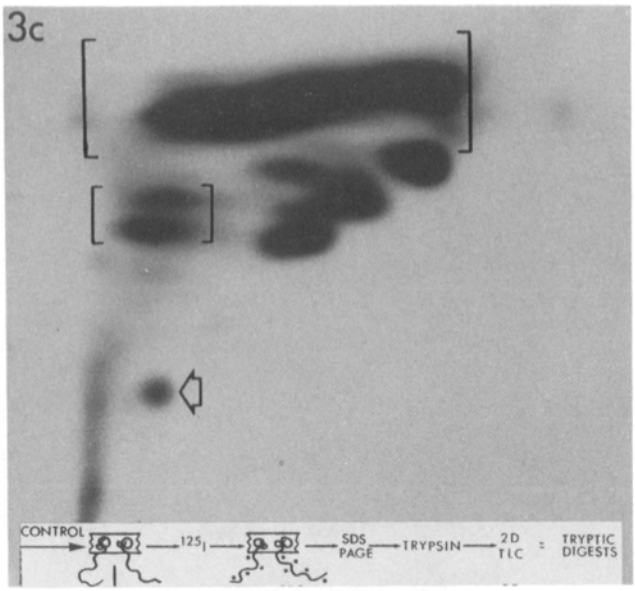
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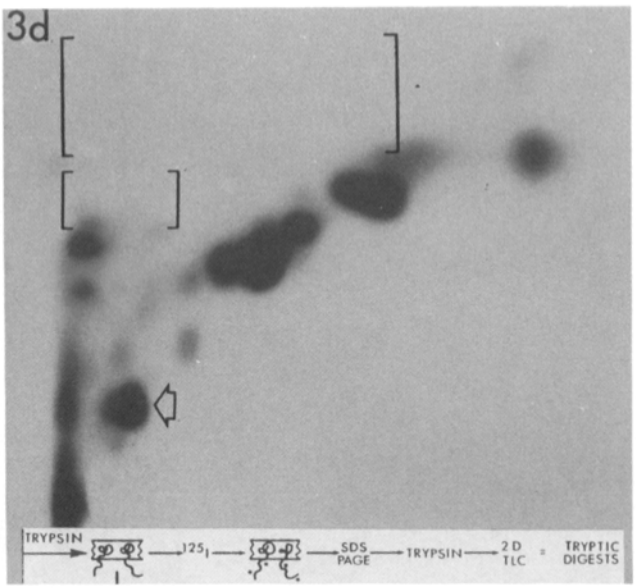
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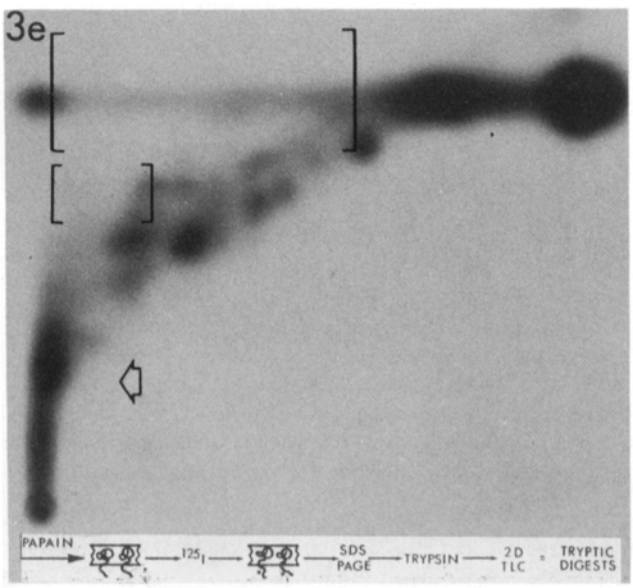
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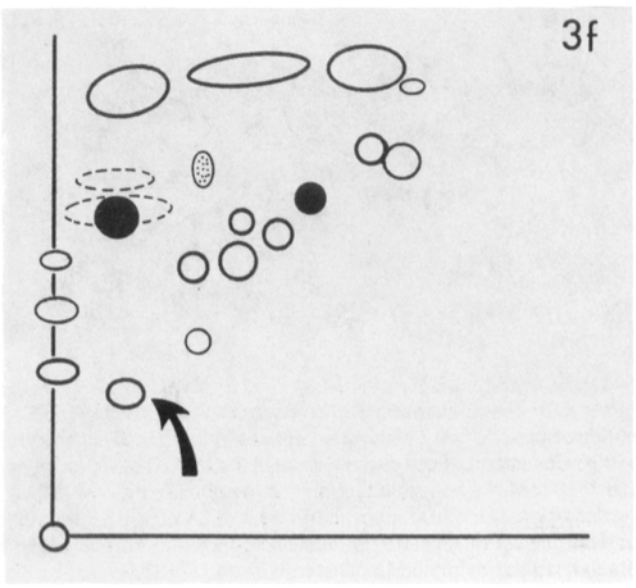
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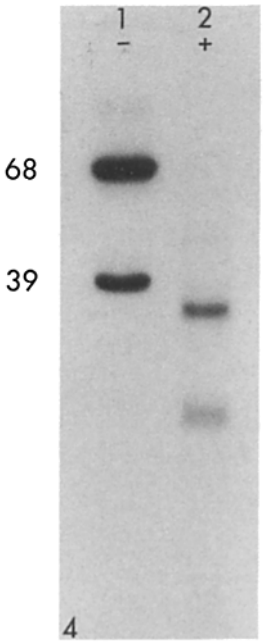


Figure 4. SDS-PAGE and autoradiography of the [^{125}I]IP39 (lane *1*) and [^{125}I]IP39$_{papain}$ (lane *2*) probes used for nitrocellulose overlays. Lane *1* is the octyl glucoside extract of radioiodinated, NaOH-stripped plasma membranes. Lane *2* is the octyl glucoside extract of NaOH-stripped plasma membranes, digested with papain, and then radioiodinated.

Methods) label was bound to the major 80-kD membrane skeletal protein but not to the equally abundant 86-kD membrane skeletal protein (Fig. 5 *b*, lanes *1* and *2*). Radiolabel was also found associated with less abundant membrane skeletal proteins (resistant to urea extraction but soluble in base, reference 14) with M_rs of ~62, 51, and 25 kD (Fig. 5 *b*, lanes *1* and *3*). Under the conditions in which these experiments were carried out little or no label was found associated with IP39 or its 68-kD dimer (Fig. 5 *b*, lane *4*), or to denatured microtubule proteins or to molecular weight standards (Fig. 5 *b*, lanes *S*, *1*, *2*) on the nitrocellulose overlays. When similar experiments were carried out with the [^{125}I]IP39$_{papain}$ probe (i.e., Fig. 4, lane *2*) no radiolabel bound to the major 80-kD membrane skeletal protein (Fig. 5 *c*, lanes *1* and *2*). The 62-, 51-, and 25-kD polypeptides, however, were radiolabeled (Fig. 5 *c*, lanes *1* and *3*).

To test the specificity of the [^{125}I] binding to the 80-kD membrane skeletal protein, a 4-M urea extract of cell surface isolates enriched in the major 86- and 80-kD membrane skeletal proteins (14) was separated by SDS-PAGE (as in Fig. 5 *a*, lane *2*) and transferred to nitrocellulose. The nitrocellulose was cut into strips and blocked with BSA and individual

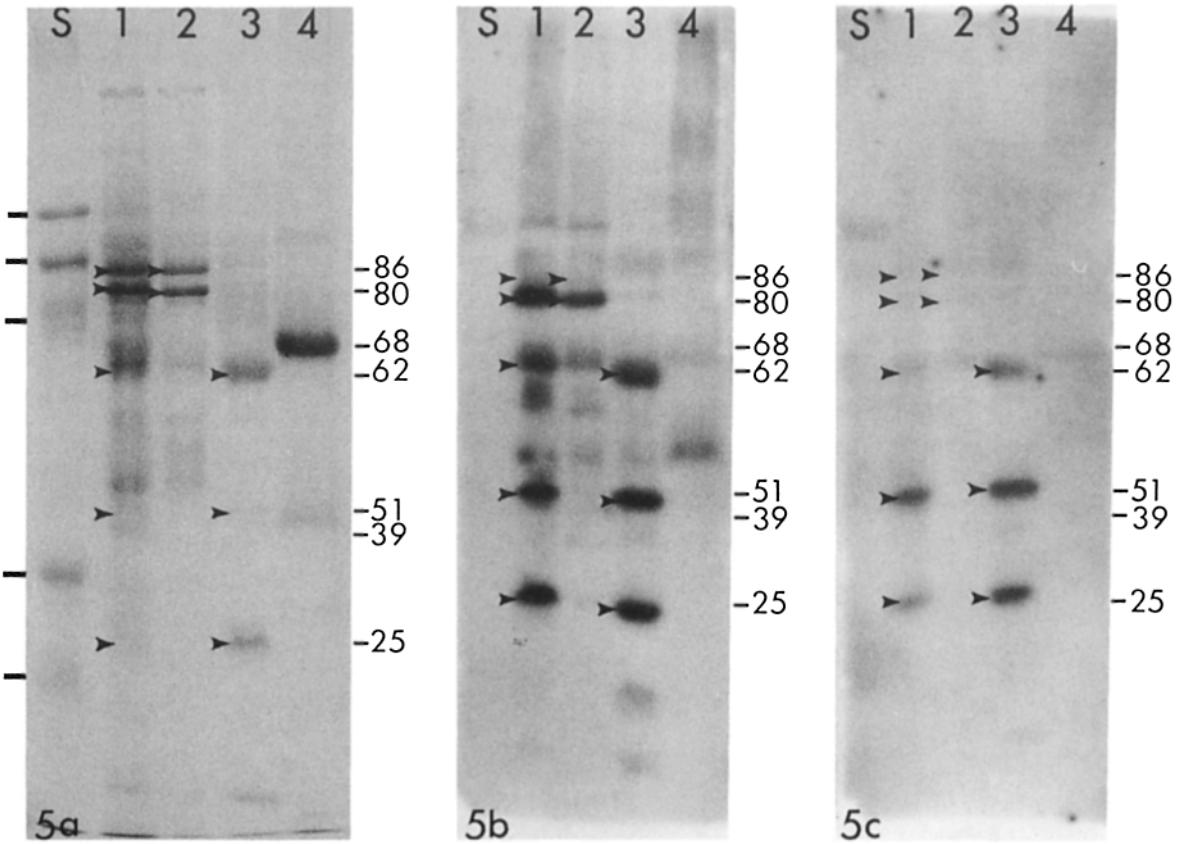


Figure 5. IP39 binds to specific membrane skeletal proteins. Three sets of surface isolates were separated on the same gel and then divided into three parts: *a* is a Coomassie brilliant blue–stained set showing molecular weight standards, lane *S*; whole cell surface isolates, lane *1*; 4 M urea extract of cell surface isolates, lane *2*; 20 mM NaOH extract of 4 M urea-resistant cell surface isolates (urea resistant proteins), lane *3*; 20 mM NaOH insoluble (integral membrane) proteins of cell surface isolates, lane *4*. Hashmarks denote molecular weight standards: β-galactosidase (116 kD), phosphorylase b (97.4 kD), BSA (66 kD), egg albumin (45 kD), carbonic anhydrase (29 kD). *b* is an autoradiograph of the set of separated proteins transferred to nitrocellulose and incubated with the [^{125}I]IP39 probe. *c* is the third set of separated proteins similar to (*b*) but incubated with the [^{125}I]IP39$_{papain}$ probe.

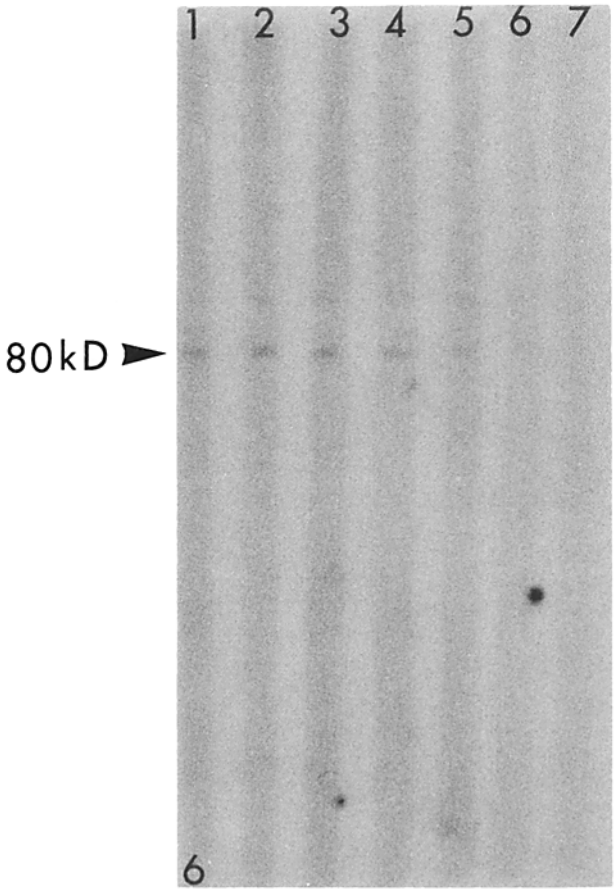


Figure 6. Unlabeled membrane extracts compete with the radioiodinated IP39 probe for binding to the major 80-kD membrane skeletal protein. A 4-M urea extract of cell surface isolates (cf., Fig. 5 *a*, lane 2) was separated by SDS-PAGE and transferred to nitrocellulose. The strips of nitrocellulose were blocked with 1% BSA and then incubated with uniform amounts of the [^{125}I]IP39 probe and increasing amounts (0×, 1×, 5×, 15×, 25×, 50×, 100×) of unlabeled, octyl-glucoside extracts of the plasma membrane (lanes *1–7*).

strips were incubated with increasing amounts of an octyl glucoside extract of unlabeled membranes (mostly IP39 and oligomers) together with a constant amount of the [^{125}I]IP39 probe. As shown in Fig. 6, [^{125}I]IP39 binding to the 80-kD membrane skeletal protein could be competitively reduced by the unlabeled membrane extract. In this experiment, the addition of a 50-fold (vol/vol) excess of unlabeled membrane extract resulted in undetectable levels of [^{125}I] in the autoradiograph.

To verify that the bound label was IP39, the 80-kD region was excised from the nitrocellulose overlay and the radiolabel was eluted with SDS-PAGE sample buffer containing 6 M urea. The eluate was then separated by SDS-PAGE and autoradiographed (Fig. 7, lane 2). It is evident that a polypeptide with a M_r identical to that of the [^{125}I]IP39 probe (Fig. 7, lane *1*) had been recovered.

Nitrocellulose transfers were stained with Ponceau S before and after incubation with radiolabeled probes. The pattern of bound proteins in both cases was identical, indicating that there was no selective loss of proteins during incubation. Furthermore, when monoclonal and polyclonal antibodies against the 86- and 80-kD membrane skeletal proteins were applied to immunoblots under similarly stringent conditions, both 80- and 86-kD polypeptides were recognized (Marrs, J. A., and G. B. Bouck, unpublished data), demonstrating that there is no preferential loss of 80- or 86-kD proteins during transfer to nitrocellulose.

Membranes without IP39 or with Papain Truncated IP39 Do Not Rebind the Major Membrane Skeletal Proteins

The major 86- and 80-kD membrane skeletal proteins can be solubilized with base or urea, and after neutralization by dialysis they will reassociate stoichiometrically on the cytoplasmic face of the plasma membrane (14). This spontaneous reassociation provides a useful assay for evaluating the role of membrane bound IP39 in anchoring or assembling membrane skeletal proteins. Flagellar membranes were prepared along with plasma membranes by base extraction of isolated flagella and cell surface isolates, respectively. Both sets of membranes were incubated with the base solubilized membrane skeletal proteins under low stringency conditions (no detergents, reference 14). In single or mixing experiments, a prominent deposition was found on the NaOH-stripped cell surface membranes (Fig. 8, *a* and *c*), whereas flagellar membranes by themselves or when mixed with plasma membranes failed to bind membrane skeletal proteins (Fig. 8 *b*). These results suggest that flagellar membranes are missing some component essential for attachment or polymerization of the membrane skeleton. Flagellar membranes lack IP39 (15) among other differences (6).

Reassociation experiments were also carried out under the stringent conditions (SDS and NP-40 present) used in the nitrocellulose overlays. A 4 M urea extract of cell surface isolates (Fig. 9, lane *2*) was incubated with plasma membranes, dialyzed against reassociation buffer, pelleted, solubilized, and then separated by SDS-PAGE. The NaOH

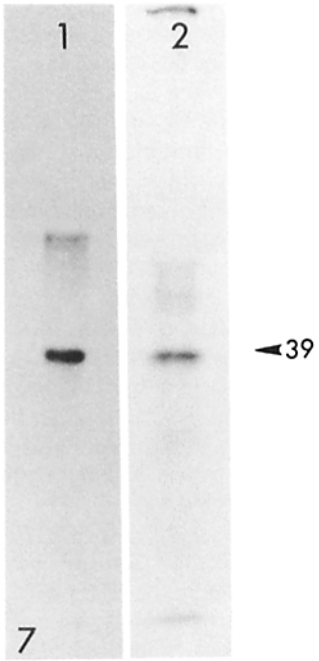


Figure 7. Radiolabeled protein recovered from the 80-kD region of a nitrocellulose overlay is [^{125}I]IP39. Lane *1* is an autoradiograph of the [^{125}I]-IP39 probe used in this experiment. Lane *2* shows the radiolabeled protein extracted from the 80-kD region of the nitrocellulose overlay.

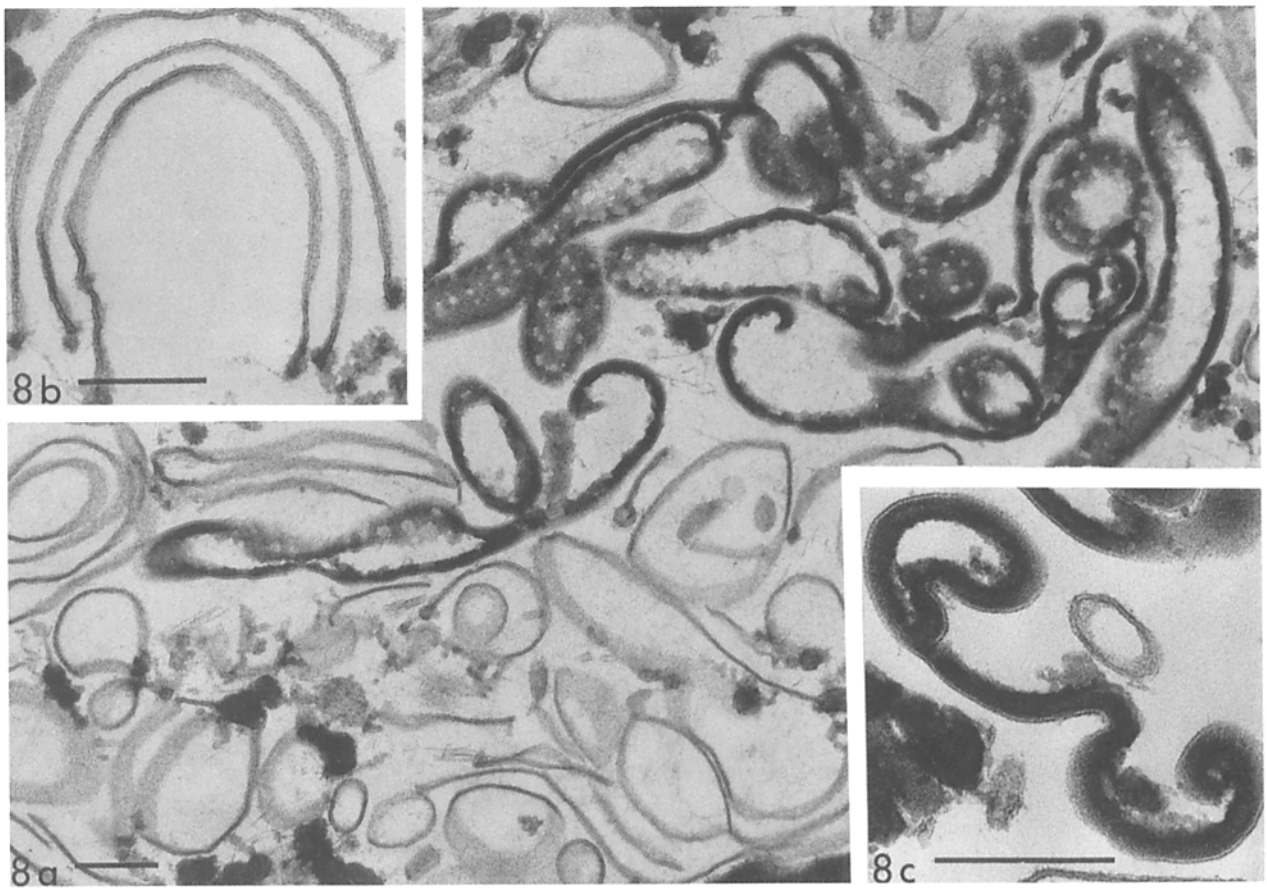


Figure 8. Reassociation in the absence of detergents of base-soluble membrane skeletal proteins with a mixture of flagellar membranes and NaOH stripped cell surface membranes (*a* and *c*) or flagellar membranes alone (*b*). Bars, 200 nm.

stripped plasma membranes were either untreated (Fig. 9, lane *3*), digested with trypsin (Fig. 9, lane *4*) or digested with papain (Fig. 9, lane *5*). The undigested plasma membranes bound significant amounts of membrane skeletal proteins (Fig. 9, lane *6*) as did the plasma membranes digested with trypsin (Fig. 9, lane *8*). A substantial amount of unbound membrane skeletal proteins remained in the supernatant in both the controls (Fig. 9, lane *7*) and when the membranes were digested with trypsin (Fig. 9, lane *9*), indicating the membrane binding was saturated or in equilibrium with unbound proteins. A mock reassociation (without membranes) showed that only very small amounts of the membrane skeletal proteins could be sedimented, i.e., aggregated spontaneously, after dialysis under these stringent conditions (Fig. 9, lane *12*). In contrast to the trypsin results when approximately equivalent amounts of NaOH stripped plasma membranes were digested with papain and then incubated with the urea extract, there was no binding of 80- and 86-kD skeletal protein (Fig. 9, lane *10*). These proteins could, however, be recovered in the unbound fraction (Fig. 9, lane *11*), indicating the failure to bind was not due to 80- and 86-kD protein degradation from residual papain activity.

Direct visual examination of similar reassociation experiments demonstrated that a membrane skeletal layer was partially reconstructed under stringent conditions (Fig. 10 *a*), although the uniformity and thickness of the layer was not as striking as when SDS and NP-40 were omitted from the reassociation buffer (Fig. 8 *c* and reference 14). To approximate the denaturing conditions under which the proteins in the nitrocellulose overlay had been separated, the NaOH soluble membrane skeletal proteins were boiled for 5 min before incubation under stringent conditions with plasma membranes. Images of these preparations (not shown) were similar to those of the unboiled extract (e.g., Fig. 10 *a*). Plasma membranes digested with trypsin and then incubated with the urea extract consistently displayed a membrane skeletal layer localized to limited areas (Fig. 10 *b*). Plasma membranes digested with papain and then incubated with the urea extract exhibited no convincing membrane skeletal layer (Fig. 10 *c*).

Discussion

Two general strategies were used to assess whether IP39 or its cytoplasmically truncated forms could mediate membrane skeletal protein interactions with the plasma membrane. First, radiolabeled octyl glucoside extracts of membrane proteins were developed as probes for an extract assay, i.e., unlabeled membrane skeletal proteins were transferred to nitrocellulose and incubated with solubilized, radiolabeled IP39; binding was assessed by autoradiography. In a second independent assay, solubilized membrane skeletal proteins were incubated with intact plasma membranes and binding was evaluated by SDS-PAGE and EM. The results of both

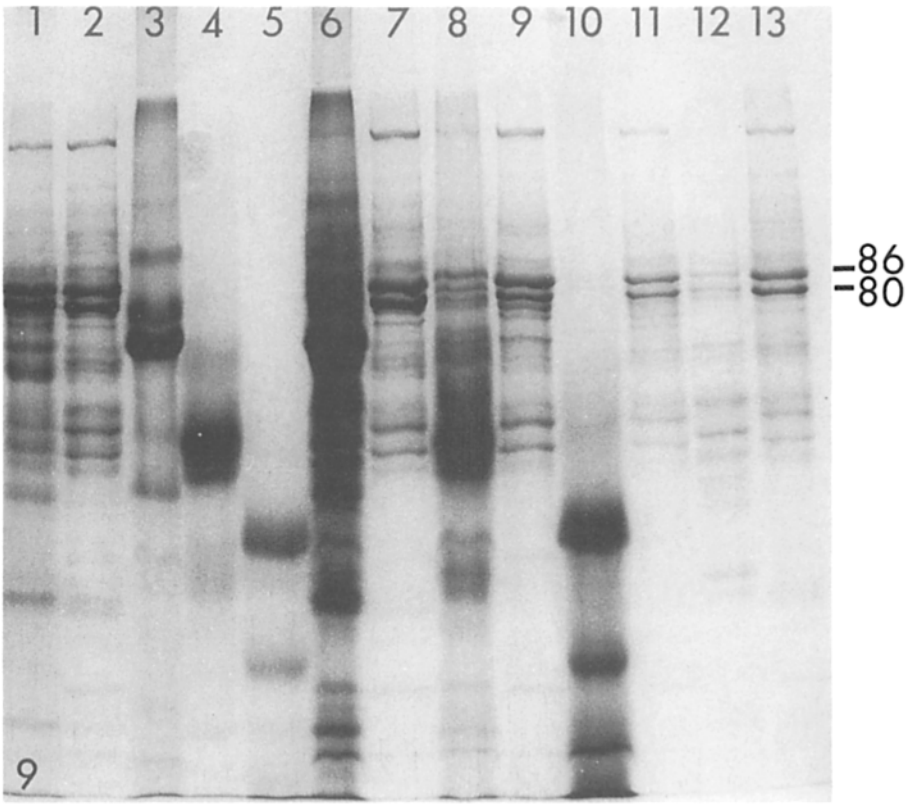


Figure 9. SDS-PAGE of membrane skeletal proteins after reassociation with undigested or trypsin- or papain-digested NaOH-stripped plasma membranes. Whole cell surface isolates, lane *1*; 4 M urea extract of cell surface isolates, lane *2*; NaOH-stripped plasma membranes, lane *3*; NaOH-stripped plasma membranes digested with trypsin, lane *4*; NaOH-stripped plasma membranes digested with papain, lane *5*. Also shown are reassociation products that sediment when the 4 M urea extract was dialyzed in the presence of undigested (lane *6*), trypsin-digested (lane *8*), or papain-digested (lane *10*) NaOH-stripped plasma membranes. Proteins not bound during the reassociation experiments using undigested (lane *7*), trypsin-digested (lane *9*), and papain-digested (lane *11*) NaOH-stripped plasma membranes are shown. The mock (no membranes) sedimented and soluble fractions are shown in lanes *12* and *13*. Lanes *5*, *10*, and *11* were from one experiment, the remaining lanes were from a separate experiment. Equivalent amounts of starting material (vol/vol) were added to each lane, but the total protein recovered varied with each treatment.

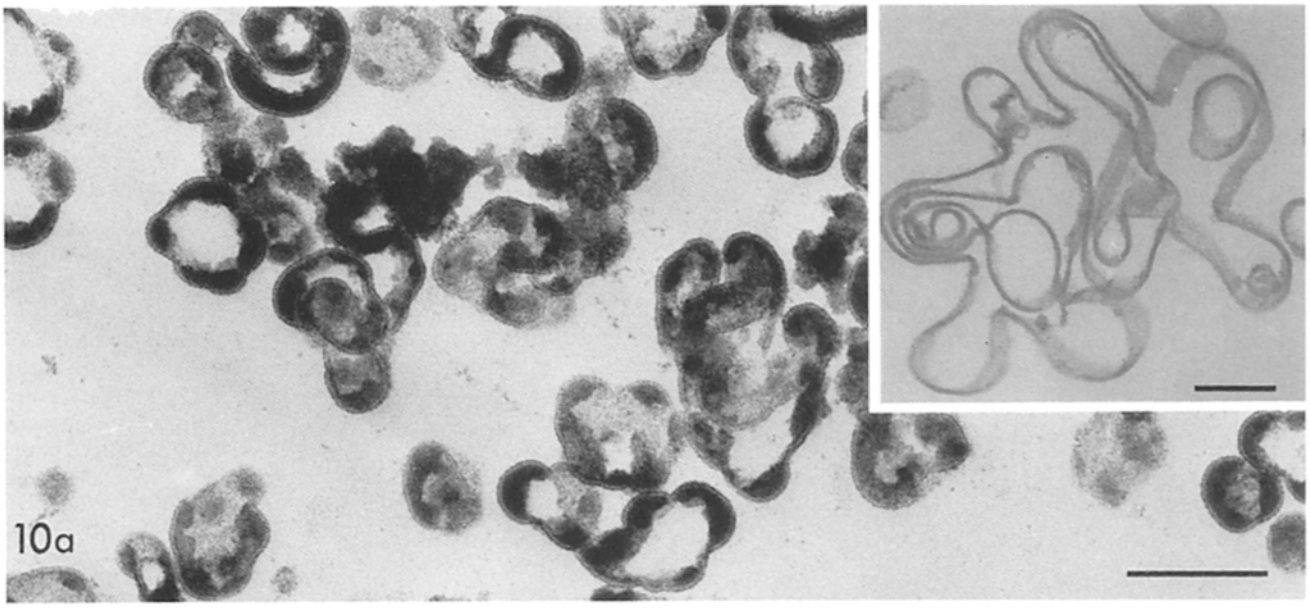
sets of experiments suggest that under the conditions tested a papain sensitive cytoplasmic region of an integral membrane protein is required for assembly of the membrane skeleton in *Euglena*.

Direct Evidence that IP39 Is the Membrane Skeletal Anchor

Detergent-solubilized radiolabeled IP39 bound to only one of the two major membrane skeletal proteins on nitrocellulose overlays. This selective binding together with its loss after portions of the cytoplasmic domain of IP39 were digested with papain suggests an important role for IP39/80-kD interactions in membrane skeletal anchorage in these cells. IP39 by itself seems to be sufficient for these interactions although it can not be ruled out that IP39 interacts with other integral membrane proteins to form a binding complex. The earlier finding that trypsin digestion of plasma membranes had no effect on membrane skeletal rebinding (14) can be reconciled with the present data in that papain removed a substantially larger portion of IP39 (Fig. 1) than trypsin. This papain-sensitive, trypsin-resistant domain of IP39 seemed to be required for membrane skeletal binding in intact membranes as well as in nitrocellulose overlays. Papain did not remove the entire cytoplasmic region of IP39 because IP39 could still be iodinated with Iodobeads following digestion of the plasma membrane. Cytoplasmic domains that are resistant to both trypsin and papain digestion could in theory provide additional binding sites for other (less abundant) membrane skeletal proteins.

The two-dimensional tryptic peptide map of the cytoplasmic regions of IP39 provided candidate domains potentially able to interact with the major proteins of the membrane skeleton. From this study it would appear that one requirement for a peptide fragment harbouring the binding domain would be its presence after trypsin digestion of IP39 in the membrane, and its absence after papain digestion. One such peptide fragment has been identified (Fig. 3, *arrows*), but further work is necessary to test whether this fragment can interact with the 80-kD proteins before this or any other peptide can be equated with the binding site. It may be of interest, however, that this trypsin-resistant, papain-sensitive fragment shown in Fig. 3, *c–e* (*arrows*) may have special properties since this iodinated peptide was also phosphorylated by an endogenous protein kinase (Rosiere, T. K., J. A. Marrs, and G. B. Bouck, unpublished observations).

The specificity of IP39 binding to the major 80-kD membrane skeletal protein is supported by evidence from both nitrocellulose overlays and reassociation experiments that showed binding was lost when cytoplasmic regions of IP39 were removed. Additionally, the [^{125}I]IP39 probe did not bind any flagellar determinants in nitrocellulose overlay experiments (not shown), as would be expected since membrane skeletal proteins did not rebind to flagellar membranes even under low stringency (no detergents) conditions (Fig. 8, *a* and *b*). This absence of flagellar binding together with previous studies showing that IP39 is absent in the flagellum (15), is consistent with a need for IP39 in membrane skeletal protein interactions. Moreover, the major membrane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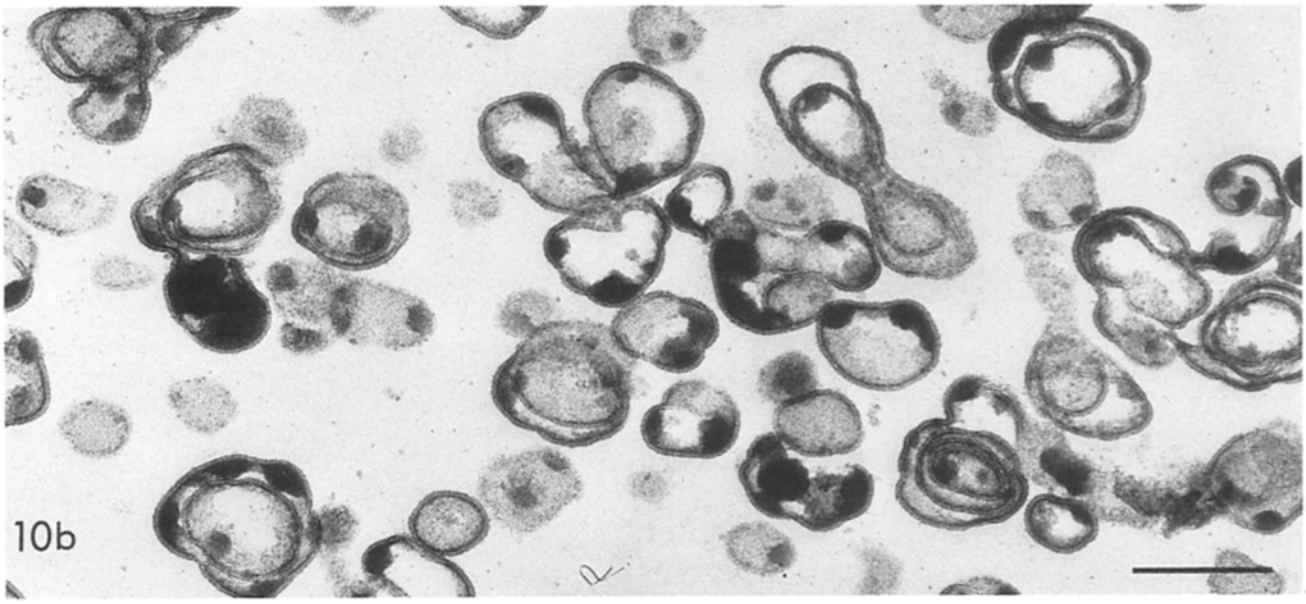
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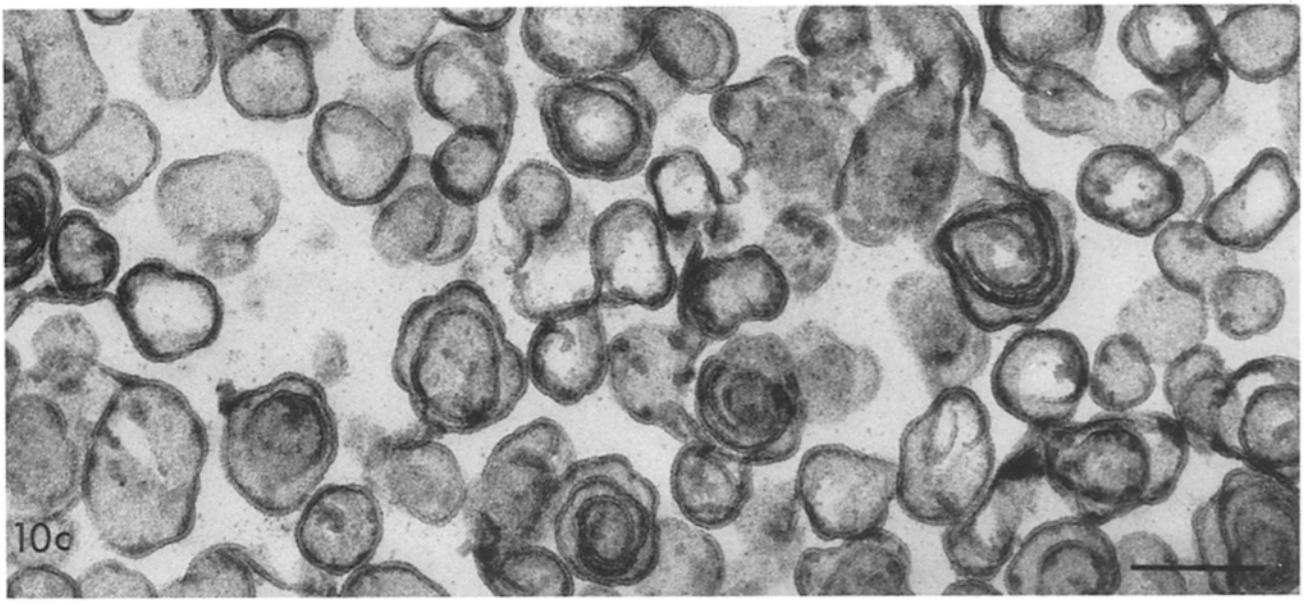
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skeletal proteins were unable to rebind to papain digested NaOH stripped plasma membranes (Fig. 9, lane *10*; Fig. 10 *c*), whereas undigested or trypsin-digested NaOH-stripped plasma membranes were reassociation competent, albeit inefficiently under these highly stringent (detergent) conditions (Fig. 9, lanes *6* and *8*; Fig. 10, *a* and *b*). It is surprising that the membrane remains visibly intact under stringent conditions, but the binding patterns were consistent in different experiments. The papain sensitivity of the 80-kD protein binding domain of IP39 strongly suggests that protein–protein interactions are required to anchor the 80-kD membrane skeletal protein to IP39 in the plasma membrane.

Soluble probes of [^{125}I]IP39 recognized one major (80 kD) and three less abundant (62, 51, and 25 kD) peripheral proteins in nitrocellulose overlay experiments (Fig. 5). In contrast to the papain sensitivity of [^{125}I]IP39 binding to the 80-kD proteins, binding to the 62-, 51-, and 25-kD polypeptides remained essentially unchanged after papain digestion. The nature of the interactions between the minor 62-, 51-, and 25-kD polypeptides and [^{125}I]IP39$_{papain}$ is unclear. (*a*) The 62-, 51-, and 25-kD peripheral proteins may associate hydrophobically with [^{125}I]IP39 or with lipids bound to [^{125}I]IP39 (for evidence of lipids associated with IP39, see reference 8). The latter would be in accord with reports of protein-lipid interactions that modulate the anchoring of some membrane skeletal proteins to the plasma membrane in other systems (1, 3, 10, 22, 23, 30, 33–35). (*b*) Those cytoplasmic regions of IP39 not removed by proteolysis of stripped membranes may contain binding sites for the 62-, 51-, and 25-kD proteins. These minor membrane skeletal proteins are resistant to extraction by 4 M urea (14) and presumably therefore have an association with the plasma membrane different from that of the urea-extractable major 80- and 86-kD membrane skeletal proteins. Conversely, the same 80- and 86-kD enriched urea extracts will reassociate with NaOH-stripped membranes (Fig. 10 *a*; reference 14), indicating that the urea-resistant, minor proteins are not required for reformation of a membrane skeletal layer.

The data presented in this paper, taken together with an earlier report of the absence of binding of the 86-kD membrane skeletal proteins after the 80 kD proteins were depleted from membrane skeletal protein fractions (14), support a general model of the association between the membrane skeletal proteins and the plasma membrane in *Euglena*. (*a*) IP39 is an anchor partially embedded in the plasma membrane. (*b*) The 80-kD membrane skeletal protein interacts directly with a cytoplasmic region of IP39. (*c*) The 80-kD membrane skeletal protein complexes with an equimolar amount of the 86-kD membrane skeletal protein. The 86-kD protein appears to be unable to bind directly to the plasma membrane. (*d*) IP39 binds the 62-, 51-, and 25-kD peripheral proteins in some manner not yet resolved. It is clear that fractions enriched in the 86- and 80-kD membrane skeletal proteins by themselves are sufficient to form the framework of a membrane skeletal layer (14) and that the role of the minor proteins remains yet to be determined.

The Membrane Anchor of Euglena Is Unique

The properties of IP39 of *Euglena* are in some ways similar to those of the erythroid protein glycophorin. These two integral membrane proteins bind membrane skeletal proteins, they have comparable molecular masses and they both can maintain subunit associations under strong denaturing conditions. Glycophorin, however, has a substantially smaller cytoplasmic domain (3 kD) (12), and polyclonal antibodies against IP39 do not cross-react in immunoblots with glycophorin or any other determinants of the human erythrocyte ghost (not shown). Moreover, IP39 and glycophorin each interact with different sets of membrane skeletal proteins. It will be of interest, nonetheless, in future studies to see if there are similarities in primary or secondary structure between IP39 and glycophorin that may reveal some conserved strategy for anchoring a membrane skeletal layer to the plasma membrane in those few cells that possess a well-defined peripheral membrane skeleton.

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Figure 10. Reassociation of membrane skeletal proteins to plasma membranes under the stringent conditions used in Figs. 5 and 9. (*a*) Undigested NaOH-stripped plasma membranes before (*inset*) and after reassociation of 4 M urea cell surface extracts. (*b*) Reassociation to NaOH-stripped plasma membranes digested with trypsin. (*c*) Reassociation to NaOH-stripped plasma membranes digested with papain. Bars, 200 nm.

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