Filopodia Are Enriched in a Cell Cohesion Molecule of Mr 80,000 and Participate in Cell–Cell Contact Formation in Dictyostelium discoideum

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Abstract. During the early phase of Dictyostelium discoideum development, cells undergo chemotactic migration to form tight aggregates. A developmentally regulated surface glycoprotein of Mr 80,000 (gp80) has been implicated in mediating the EDTA-resistant type of cell cohesion at this stage. We have used a monoclonal antibody directed against gp80 to study the topographical distribution of gp80 on the cell surface. Indirect immunofluorescence studies showed that gp80 was primarily localized on the cell surface, with a higher concentration at contact areas. Immunoelectron microscopy was carried out by indirect labeling using protein A-gold, and a nonrandom distribution of gp80 was revealed. In addition to contact regions, gold particles were found preferentially localized on filopodia. Quantitative analysis using transmission electron microscopy (TEM) showed that ~60% more gold particles were localized in contact regions in comparison with the noncontact regions, and the filopodial surfaces had a twofold higher gold density. Both TEM and scanning electron microscopy showed that contact areas were enriched in filopodial structures. Filopodia often appeared to adhere to either smooth surfaces or similar filopodial structures of an adjacent cell. These observations suggest that the formation of stable cell–cell contacts involves at least four sequential steps in which filopodia and gp80 probably play an important role in the initial stages of recognition and cohesion among cells.

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Since the monoclonal antibody used in their study has cross-reactivity with other cellular components (22), it may recognize other surface molecules besides gp80, thus complicating the interpretation of their results.

In this report, we have used the monoclonal antibody 80L5C4 and investigated the topographical distribution of gp80 on the cell surface. Indirect immunolabeling using protein A-gold reveals the nonrandom distribution of gp80. Quite unexpectedly, gp80 has been found to preferentially cluster on filopodia and other tubular projections in addition to the contact surfaces. Filopodial structures are often found in abundance between two cells in close apposition and they probably play an important role in the initial stages of contact formation.

Materials and Methods

Cell Cultures

Dicyostelium discoideum strains NC4 and V12/M2 were cultured in association with Klebsiella aerogenes and were developed as described (27). Growth phase cells were washed free of bacteria. Cells (10⁷/ml) were developed for 9 h on filter paper (No. 50; Whatman, Inc., Clifton, NJ) to obtain cells in their midstreaming stage. Alternatively, cells were developed at 10⁶ cells/ml for 12 h in PDF, a starvation medium (27), and pulsed with 2 × 10⁻⁵ M cAMP at 8-min intervals. Cells were deposted on 2% plain agar or tissue culture plastic to allow cell streaming to occur.

Monoclonal Antibodies

Two murine monoclonal antibodies, 80L5C4 and 80L7B6, were used. Production and characterization of these two monoclonal antibodies are described elsewhere (24). Monoclonal antibody 80L5C4 reacts only with gp80, while monoclonal antibody 80L7B6 recognizes the carbohydrate moiety on gp80 and it cross-reacts with a number of glycoproteins.

Preparation of Protein A-Gold

Colloidal gold conjugate was prepared according to the method of Frens (11). Protein A-gold was prepared essentially according to the procedures of De Mey (8) and Bendayan (1). To obtain colloidal gold particles of 40 nm in diameter, 1 ml of 1% sodium citrate was added to 100 ml of boiling 0.01% tetrachloroauric acid. To prepare 20 nm colloidal gold, 3 ml of 1% sodium citrate was used. The solution was kept boiling until the color changed from pale yellow to wine red. Upon cooling to room temperature, protein A was adsorbed to the gold colloid at pH 6.9 (1). The protein A-gold particles were washed with Dulbecco's PBS containing 0.1% polyethylene glycol of Mr 10,000. Gold conjugates were washed free of excess protein A by centrifugation for 30 min at 75,000 g for 40 nm gold and 20,000 g for 20 nm gold, each for 30 min. The washed gold probes were stored in 1 ml of PBS containing 0.1% polyethylene glycol and 20 mM sodium azide. The 20-nm gold probes were used for transmission electron microscopy (TEM), while the 40-nm were used for scanning electron microscopy (SEM).

Immunofluorescence Microscopy

Development of cells was carried out in 17 mM sodium phosphate buffer, pH 6.5, at 1 × 10⁷ cells/ml for 12 h. Then the cells were deposited on a tissue culture dish and allowed to form cell streams. After fixation with methanol for 10 min at −20°C, cells were washed with 0.1% BSA in PBS, stained with anti-gp80 monoclonal antibody and TRITC-conjugated goat anti-mouse IgG antibodies. Cells were mounted in 2% n-propyl gallate in glycerol, pH 7.2, as described by Giloh and Sedat (13). Epifluorescence was observed using a Wild Leitz Orthoplan universal largefield microscope.

Transmission Electron Microscopy

Aggregation stage cells were labeled with monoclonal antibody with or without prior fixation in 0.5% glutaraldehyde in 25 mM cacodylate buffer, pH 7.2, for 5 min at room temperature. In case of postfixation, cells were washed with 100 mM glycine and then labeled with monoclonal antibody 80L5C4 and protein A-gold. Cells were washed with 0.1% BSA and then with PBS before postfixation with 2% glutaraldehyde for 60 min, and then with 1% osmium tetroxide for 30 min at room temperature (28). The sample was dehydrated in a graded series of ethanol, embedded in Spurr's low viscosity medium, and sectioned. After staining with aqueous uranyl acetate (5%; 20 min) and lead citrate (0.01%; 1 min), the sections were observed using a Philips 300 transmission electron microscope.

Quantitative Analysis

The distribution of colloidal gold particles was analyzed by measuring their number on the cell surface contour of EM micrographs. The surface of each cell profile was divided into three main regions: contact surfaces between cells, noncontact smooth surfaces, and filopodial structures. Filopodial structures included circular profiles of 0.3 μm or less in diameter and tubular structures of similar diameter, with a minimum of 0.3 μm protruding from the cell surface. Random cell profiles were blown up to a uniform magnification for analysis. The length of each cell surface region was measured using a computer-aided Apple Bioquant Tablet. The number of gold particles in each category was counted manually. In case of a particle located between two membranes in close apposition, a value of 0.5 particle was assigned to each membrane. Raw data were entered in a per cell basis. Statistical analysis was performed using the nonparametric method of Mann and Whitney (25). The Student's t-distribution was consulted after the critical value was tabulated for each pair of data sets under evaluation. The probability levels at which significant differences in gold densities between the contact and noncontact (excluding filopodial structures) areas and between the filopodial and noncontact regions occurred were determined.

Scanning Electron Microscopy

Cells were deposited on coverglass coated with 0.1% poly-l-lysine (19). After 7-8 h of development, cells were prefixed with 0.25% glutaraldehyde in 25 mM cacodylate buffer, pH 7.2, for 5 min and then washed with 0.1% glycine. Cells were labeled with the monoclonal antibody 80L5C4 and protein A-gold after postfixation with 2.5% glutaraldehyde for 30 min. Ethanol dehydration was followed by critical point drying using liquid CO₂. Preparations were mounted on metal stubs and coated with carbon. Samples were observed with a Jeol JSM 840 scanning electron microscope, using secondary electron imaging (SEI) and backscattering electron imaging (BEI) modes. Gold particles appeared as white images when normal polarity was used in the BEI mode. When the polarity was reversed, gold particles appeared as black dots. Signals from the SEI and BEI modes, set at normal polarity, were also mixed to generate a compositional image according to De Harven et al. (7).

Results

Surface Localization of gp80 by Immunofluorescence Microscopy

The monoclonal antibody 80L5C4, which was previously shown to be monospecific for gp80 (24), was used to determine its subcellular localization. Aggregation stage cells were fixed on a tissue culture dish and stained indirectly using a TRITC-conjugated second antibody. Fluorescence was primarily associated with the plasma membrane and there was little staining of the cytoplasm. When cell aggregates were examined, much brighter fluorescence was localized at the contact zones between neighboring cells (Fig. 1 a and b). Fluorescence was often observed at end-to-end contacts of cells in a streaming pattern. Occasionally, bright fluorescent spots were seen on cell protrusions, suggesting the occurrence of gp80 clusters on these membrane structures. It was also of interest to note that migrating cells were more elongated, while cells in aggregates had few pseudopods and were more rounded in shape.

When live cells were labeled with monoclonal antibody 80L5C4, fluorescent labeling of the cell surface became evident (Fig. 1, c and d). Cap formation was observed when
cells were incubated at room temperature for 20 min or more. These results show that gp80 is exposed on the cell surface and it is capable of lateral movement in the plasma membrane. When cells from the vegetative stage or the preaggregation stage were labeled in the same way, no fluorescence was detected on the cell surface, indicating that the appearance of gp80 on the plasma membrane is developmentally regulated.

To demonstrate that the surface staining of monoclonal antibody 80L5C4 was specific, fluorescent staining was also performed with another monoclonal antibody, 80L7B6, which recognizes a carbohydrate epitope on gp80 and thus cross-reacts with a number of other glycoproteins (24). In contrast to the localized staining patterns obtained with 80L5C4 IgG, fairly uniform staining of the cytoplasm, as well as the plasma membrane, was observed with 80L7B6.
Clustering of gp80 at Contact Regions

Labeling of aggregating cells using TRITC-conjugated second antibody suggested that gp80 was preferentially associated with contact areas. To visualize contact zones at higher magnification, cells were examined by TEM. Aggregation stage cells were briefly prefixed with glutaraldehyde before labeling with monoclonal antibody 80L5C4 and protein A-gold. Fig. 2 a shows the distribution of gold particles, indicative of the location of gp80, on the cell surface. The control, using nonimmune mouse IgG, showed no nonspecific binding or trapping of the protein A-gold (Fig. 2 b).
Figure 3. Localization of gp80 in contact regions of aggregating cells. (a) 9-h NC4 cells were developed on filter paper, prefixed with 0.5% glutaraldehyde for 5 min, labeling with monoclonal antibody 80L5C4, protein A-gold, and processed for TEM. (b and c) Liquid developed 12-h NC4 cells, without prefixation, were labeled with monoclonal antibody 80L5C4 followed by protein A-gold. Cells were then fixed and processed for TEM as described in Materials and Methods. (d) Control: 12-h cells were labeled with nonimmune mouse IgG before fixation for EM. Bars: (a) 0.25 μm; (b) 0.25 μm; (c) 0.5 μm; (d) 0.5 μm.
Figure 4. Clustering of gp80 on filopodia and surface ridges. Aggregation stage NC4 cells were labeled with monoclonal antibody 80L5C4 followed by protein A-gold. (a) Cells fixed before antibody labeling. (b and c) Cells labeled without prior fixation. Bars: (a) 0.5 μm; (b) 0.25 μm; (c) 0.25 μm.

Staining patterns using either protein A-gold or second antibody-gold conjugates were also compared and the results showed no significant difference.

A relatively higher density of gold particles was found in areas of initial contact formation (Fig. 3 a), which were usually characterized by limited membrane alignment between two cells and many cross-sections of tubular structures, with a diameter of 0.1-0.2 μm. Gold particles were much lower in areas of extensive cell contact where membranes of two adjacent cells showed close and parallel alignment. Precipitation of cells had probably rendered the gp80 in these membrane zones inaccessible to the monoclonal antibody and/or protein A-gold. Live cells were, therefore, labeled with monoclonal antibody at low temperature followed by glutaraldehyde fixation. Results showed that the general labeling pattern was similar to that of prefixed cells except that clusters of gold particles became detectable in regions of extensive membrane contact (Fig. 3 b and c). In the control where nonimmune mouse IgG was used, trapping of gold particles in the contact region was negligible (Fig. 3 d).

Preferential Localization of gp80 on Filopodia

Preferential association of gold particles with tubular structures projecting from the cell surface was evident from a number of micrographs (Fig. 4). Many of these cellular ex-
Table I. Distribution of gp80 on Cells at the Aggregation Stage

<table>
<thead>
<tr>
<th>Subregions of contour</th>
<th>No. of gold particles</th>
<th>Contour length</th>
<th>Density</th>
<th>Relative density*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μm</td>
<td>gold particles/10 μm</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1855</td>
<td>1277</td>
<td>14.4</td>
<td>1.31</td>
</tr>
<tr>
<td>II</td>
<td>620</td>
<td>581</td>
<td>10.7</td>
<td>0.94</td>
</tr>
<tr>
<td>III</td>
<td>3256</td>
<td>624</td>
<td>52.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Noncontact region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>849</td>
<td>772</td>
<td>11.0</td>
<td>1.00</td>
</tr>
<tr>
<td>II</td>
<td>443</td>
<td>388</td>
<td>11.4</td>
<td>1.00</td>
</tr>
<tr>
<td>III</td>
<td>1866</td>
<td>336</td>
<td>55.5</td>
<td>1.00</td>
</tr>
<tr>
<td>Cell-cell contact region</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>498</td>
<td>283</td>
<td>17.6*</td>
<td>1.60</td>
</tr>
<tr>
<td>II</td>
<td>78</td>
<td>144</td>
<td>5.4</td>
<td>0.45</td>
</tr>
<tr>
<td>III</td>
<td>295</td>
<td>80</td>
<td>36.9**</td>
<td>0.66</td>
</tr>
<tr>
<td>Filopodia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>508</td>
<td>222</td>
<td>22.9†</td>
<td>2.08</td>
</tr>
<tr>
<td>II</td>
<td>99</td>
<td>49</td>
<td>20.2**</td>
<td>1.77</td>
</tr>
<tr>
<td>III</td>
<td>1095</td>
<td>208</td>
<td>52.6</td>
<td>0.95</td>
</tr>
</tbody>
</table>
* The relative density of each area is normalized as a ratio to the gold density of the noncontact region in each experiment.
† Live NC4 cells (9 h) were labeled with monoclonal antibody 80LSC4 and protein A-gold, followed by fixation with glutaraldehyde as described in Materials and Methods. 60 random cell profiles of similar dimension were used in this analysis.
‡ Cells were prefixed in 0.5% glutaraldehyde for 5 min at room temperature before labeling with 80LSC4 IgG and protein A-gold. 30 random cell profiles were analyzed.
§ Cells were labeled with Con A for 10 min at 4°C, followed by rabbit anti-Con A antibody and protein A-gold. After several washes, cells were fixed with glutaraldehyde for EM. 30 random cell profiles were analyzed.
¶ Significantly different from the density in the noncontact region of I, with \( P < 0.05 \) (Mann-Whitney test).
** Significantly different from the density in the noncontact region of II, with \( P < 0.05 \) (Mann-Whitney test).
†† Significantly different from the density in the noncontact region of I, with \( P < 0.001 \) (Mann-Whitney test).
§§ Significantly different from the density in the noncontact region of II, with \( P < 0.05 \) (Mann-Whitney test).

Tensions could be attributed to filopodia that were present on cells in large numbers at the aggregation stage (6). Filopodia were often seen in abundance at regions where two cells were in close apposition to each other (Fig. 5). Both cross-sections or tangential sections of filopodia showed an enrichment in gp80. In some sections, tubular protrusions with diameters larger than those of the average filopodia were also observed (Fig. 5, b and c). These structures might represent pseudopods transformed from filopodia (26). In some cell-cell contact regions, interdigitation of filopodia was evident, with clusters of gold particles associated with filopodia and the plasma membrane (Fig. 5 d).

Quantitative Analysis of gp80 Distribution

The distribution of gp80 on the cell surface was subjected to quantitative analysis. The gold density in different regions of the plasma membrane was estimated in two different preparations. The plasma membrane was sub-divided into three major categories: (a) noncontact region, (b) contact region, and (c) filopodia as described in Materials and Methods. In the first experiment, aggregation-stage cells were labeled with monoclonal antibody 80LSC4 and protein A-gold at low temperature before fixation. The average gold density in the noncontact region was 11 particles/10 μm. The gold particle densities in the contact region and on the filopodia were 18 and 23 particles per 10 μm, respectively (Table I). By normalizing against the gold density at the noncontact region, a twofold increase in gold particles/unit length was found associated with the filopodia and the gold density was 60% higher for the contact region. The differences in the gold density between the filopodia or contact zones and the noncontact surfaces were shown to be statistically significant by the Mann and Whitney test. In the second experiment, cells were prefixed briefly with glutaraldehyde before antibody labeling. A slight general decrease in gold labeling on the cell surface was observed. An analysis of the density of gold particles in the three different regions again revealed a biased distribution of gp80 (Table I). Few particles were observed at the cell-cell contact zones, probably because accessibility to these regions was blocked by glutaraldehyde prefixation. As a positive nonspecific control, cells were labeled with concanavalin A (Con A), followed by rabbit anti-Con A antibody, and protein A-gold. The average density of gold particles was fivefold higher than 80LSC4 labeling. Analysis of the distribution of gold particles showed no significant difference between the noncontact region and the filopodia. However, the density was about one-third lower in the contact region (Table I).

Participation of Filopodia in Cell-Cell Interaction

Results from the above TEM studies suggest that filopodia are involved in the formation of cell-cell contacts. This was further investigated by SEM, which allowed us to examine whole cells instead of thin sections. Cells that were not yet in the streaming trail exhibited a large number of filopodia on the surface (Fig. 6). Filopodia clusters were often observed in areas where cells were in close apposition. Many
Figure 6. Scanning electron micrographs of 7-h migrating cells. NC4 cells were developed in liquid medium and pulsed with $2 \times 10^{-8}$ M cAMP for 6 h. Cells were then deposited on a tissue culture dish and developed for 1 h to allow cell streaming to take place. Cells were processed for SEM as described in Materials and Methods. (a) Two cells in close apposition, and (b) cells showing the participation of filopodia in cell-cell interaction. (a) Picture taken at 15 kV; bar, 5 μm. (b) Picture taken at 5 kV; bar, 5 μm.
of them appeared to be adhering to the surface or filopodia of the adjacent cell. Cells in migrating streams were usually more elongated and most of the filopodia were found at the polar ends with only a few on the lateral surfaces (Fig. 8 a). Interdigitation of filopodia were often observed where two cells were making end-to-end contacts. Cells that had established end-to-end contacts appeared to have retracted most of their pseudopods and filopodia.

When the specimen was observed at a lower accelerating voltage, prominent ridges of membrane folds became evident (Fig. 6 b). These parallel ridges were characteristic of streaming cells at the aggregation stage.

**Correlation of gp80 Distribution with Surface Topography Using SEM**

SEM was used to further investigate the association of gp80 with cell surface structures at the cell contact zones. Recently, a novel SEM technique was introduced by de Harven et al. (7) whereby the locations of antigens labeled with immunogold can be correlated with the surface topography of the cell. This is achieved by mixing the SEI and BEI signals to produce compositional images that show both cell morphology and gold particles. Fig. 7 shows the filopodial activity between two neighboring cells, which were probably in the initial stage of making cell–cell contacts. The filopodia were heavily labeled with gold particles. Although it was difficult to quantitate the distribution of gold particles in these images, the filopodia appeared to have a relatively higher density of gold particles. It was also of interest to note that a great proportion of gold particles on the cell surface was associated with the ridges of membrane folds. Fig. 8 shows a cell that appeared to be in the process of forming end-to-end contacts with another cell. Filopodia were present primarily at its two polar ends. At one end, the filopodia
interdigitated with those of another cell. When the BEI mode was used to examine this region at a higher magnification, intense gold labeling was found at the contact zone (Fig. 8 b). When viewed with the mixed imaging mode, the association of a substantial number of gold particles with the filopodial surface and membrane ridges became obvious (Fig. 8 c).

**Discussion**

Using a monoclonal antibody that is monospecific for gp80, we have demonstrated by immunofluorescent-labeling studies that gp80 is primarily localized on the cell surface. This is consistent with our previous observation that mild proteolytic digestion of intact cells can remove essentially all of the cellular gp80 (17), indicating that gp80 is exposed on the cell surface. Quantitative measurement of gp80 expression also shows that >95% of the endogenous gp80 is associated with the cell surface (24; unpublished results).

Both immunofluorescent labeling and immuno-EM studies showed that gp80 has a nonrandom distribution on the cell surface. gp80 molecules are more abundant at the cell-cell contact regions. Quantitative analysis indicates the density of gp80 molecules in cell contact regions is at least 60% higher than that of the smooth noncontact regions. The relative abundance of gp80 in the contact zones is likely to be underestimated. Since monoclonal antibody 80L5C4 is capable of blocking cell-cell binding (24), binding of the antibody to gp80 can only be achieved by competing for the cell-binding site on gp80 and disrupting the interaction between gp80 and its receptor. In addition, the close alignment between membranes in the contact zone may render the contact surfaces less accessible to IgG and protein A-gold. Indeed, in the nonspecific positive control labeled with Con A, there was a significant decrease in gold density in the contact region although no difference was observed for the noncontact region and the filopodia (Table I). In addition, a higher density of gold labeling is often observed in regions right at the two ends of the cell junction, suggesting that the concentration of gp80 within the contact zone could be higher than that revealed by the protein A-gold. In fact, this pattern of distribution is similar to that of gp150 (14), which has been implicated in mediating the EDTA-resistant cell-cell binding sites at the postaggregation stages (16, 23). Thus, the plasma membrane appears to be organized in domains enriched in components of specialized function such as cell cohesion molecules. It is not clear whether these domains exist before contact formation or during the process of contact formation.

The most interesting observation revealed by our TEM studies is the preferential association of gp80 with the filopodia. A twofold enrichment of gp80 can be observed on these slender and tubular structures. SEM studies further confirmed a biased distribution of gp80 on the cell surface. In addition to filopodia, SEM revealed a preferential association of gold particles with the ridges of membrane folds. The significance of this observation is not clear. It is conceivable that the membrane folds and filopodia may be related structurally or in their formation since the appearance of these morphological structures is unique to cells at the aggregation stage of development. Filopodia begin to appear in large numbers after 4-5 h of starvation (6). They are dynamic structures and they are formed and withdrawn from motile cells in response to environmental stimuli (26). When cells become elongated in their active migration stage, filopodia are found predominantly at the polar ends and tips of pseudopods, and they become greatly reduced in number after stable cell-cell contacts are formed.

The increase in filopodia and changes in cell shape will require an increase in cell surface area and thus membrane biosynthesis. At the onset of the cell aggregation stage, a rapid translocation of phospholipid to the plasma membrane occurs via lipid-rich vesicles (9, 10), and these newly synthesized membranes are probably involved in the formation of filopodia. Lipid transport and plasma membrane expansion are not observed in certain aggregateless mutants (9). Analysis of several groups of aggregateless mutants by Chastellier and Ryter (6) has led to the conclusion that the formation of filopodia is a necessary step for cell aggregation and it can be stimulated by cAMP pulses.

Our EM studies suggest that filopodia may play an important role in the formation of end-to-end contacts between cells. Although it is difficult to reconstruct the dynamic activities leading to contact formation, based on a large number of TEM and SEM micrographs one can envisage at least four stages of cell-cell interaction (Fig. 9). In the first stage, aggregation competent cells send out filopodia to establish initial contacts with adjacent cells. It can be viewed as a recognition step by which a cell seeks out its partner for further interaction. In the second stage, the number of filopodia
from both cells appear to proliferate at the contact zone and they interdigitate with each other. Such intimate interactions among the filopodia probably help to draw the two cells into closer contact. In the third stage, filopodia at the contact region are withdrawn by both cells, leading to the final stage whereupon stable contacts are formed between smooth membrane surfaces. Although filopodia are clearly involved in end-to-end cell contacts, they are not restricted to the polar ends of an elongated cell. It is not clear to what extent, if any, they are involved in the side-to-side contact formation that is presumably mediated by the EDTA-sensitive binding sites (15). A similar approach using antibodies directed against the EDTA-sensitive contact sites (5) will help to determine their surface localization and their relationship with gp80.

Gp80 shows preferential association with filopodia, and it is likely that gp80 is involved in the initial recognition and interaction between cells. The plasma membrane at this stage can be visualized as a very active organelle, constantly retracting and sending out new filopodia and pseudopods in its interaction with adjacent cells and in response to exogenous cAMP. It is conceivable that the cytoskeleton plays an important role in the formation of filopodia and in the lateral movement of gp80 in the plasma membrane. However, little is known about the interaction of gp80 with other membrane components or cytoskeletal elements. The availability of both immunological and molecular probes (29) should provide us with some useful tools to address these issues in the future.

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