Cytoskeleton Interactions Involved in the Assembly and Function of Glycoprotein-80 Adhesion Complexes in Dictyostelium*

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Adhesion complexes typically assemble from clustered receptors that link to the cytoskeleton via cytoplasmic adapter proteins. However, it is unclear how phospholipid-anchored adhesion molecules, such as the Dictyostelium receptor gp80, interact with the cytoskeleton. gp80 has been found to form adhesion complexes from raftlike membrane domains, which can be isolated as a Triton X-100-insoluble floating fraction (TIFF). We report here that the actin-binding protein ponticulin mediates TIFF-cytoskeleton interactions. Analysis of gp80-null cells revealed that these interactions were minimal in the absence of gp80. During development, gp80 was required to enhance these interactions as its adhesion complexes assembled. Whereas ponticulin and gp80 could partition independently into TIFF, gp80 was shown to recruit ponticulin to cell-cell contacts and to increase its partitioning into TIFF. However, these proteins did not co-immunoprecipitate. Furthermore, sterol sequestration abrogated the association of ponticulin with TIFF without affecting gp80, suggesting that sterols may mediate the interactions between ponticulin and gp80. In ponticulin-null cells, large gp80 adhesion complexes assembled in the absence of ponticulin despite the lack of cytoskeleton association. We propose that such nascent gp80 adhesion complexes produce expanded raftlike domains that recruit ponticulin and thereby establish stable cytoskeleton interactions to complete the assembly process.

Dynamic tissue rearrangements are fundamental to both embryonic morphogenesis and adult processes such as stem cell development, immune responses, and cancer progression (1). These rearrangements involve the coordination of cell-cell adhesion with cytoskeleton-based cell migration and cell shape changes. Thus, cytoskeleton interactions with adhesion complexes are probably of critical importance. We have used Dictyostelium as a model system to examine interactions between adhesion complexes and the actin cytoskeleton during multicellular development. Dictyostelium has a simple and well-defined life cycle that permits biochemical and genetic analyses of cell-cell interactions (2). During the aggregation stage of development, Dictyostelium undergoes a transition from individual cells to multicellular streams. The process begins with the chemotactic migration of single cells toward a central source of cAMP (2). Cell-cell contacts are initially established through the interdigitation of filopodia (3). The contacts break and reform continually as the cells migrate to the aggregation center (4). Even within large migratory multicellular streams, individual cells form only transient cell-cell contacts and continually exchange partners (5).

In general, cell-cell adhesion is mediated by large adhesion complexes composed of a core of adhesion molecules and peripheral attachments to the cytoskeleton (6). Within the complexes, cis- and trans-oligomers of adhesion molecules can assemble into zippers and lattices (7–9), structures that can convey strong cell-cell adhesion through enhanced binding avidity (10). The adhesion receptors are typically connected to the cytoskeleton via adapter proteins, such as the catenins that link cadherin complexes to the actin cytoskeleton (11). In Dictyostelium, cell-cell contacts are first formed by the Ca2+-dependent adhesion molecule DdCAD-1 (12–14) and distinct Mg2+-dependent adhesion sites (15). The Ca2+/Mg2+-independent adhesion molecule gp80 is expressed at the onset of the chemotactic migration stage (16) and mediates strong cell-cell adhesion (17, 18) via trans-homophilic interaction (19, 20). The expression of the next major cell adhesion molecule gp150 is activated at the cell aggregation stage (21), and rapid accumulation of gp150 occurs in the postaggregation stage (22).

gp80 is phospholipid-anchored (23, 24), as are many other adhesion molecules such as NCAM-120, axonin-1/TAX-1/TAG-1 (25), and T-cadherin (26). Since these receptors lack direct access to the cytoplasm, the mechanism by which they engage the cytoskeleton is unclear. Many glycosylphosphatidylinositol (GPI)1-anchored proteins preferentially partition into rafts, which are membrane microdomains formed from the close packing of sterols and saturated lipids into liquid ordered structures (27). Rafts can be isolated from cells as Triton X-100-insoluble floating fractions (TIFF) that contain the raft lipids, as well as GPI-anchored proteins, acylated proteins, and some transmembrane proteins (28–30).

Raftlike membrane fragments have been isolated from Dictyostelium as both TIFF and a low density plasma membrane fraction after sonication. Both types of membrane fractions were highly enriched in sterols and contained many of the proteins found in the bulk plasma membrane, suggesting that raftlike domains exist in the Dictyostelium plasma membrane (31, 32). Moreover, immunoprecipitation experiments have

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¶¶ The abbreviations used are: GPI, glycosylphosphatidylinositol; MALDI-TOF, matrix-assisted laser desorption and ionization time-of-flight; TIFF, Triton X-100-insoluble floating fraction; TISF, Triton X-100-insoluble sedimenting fraction; mAb, monoclonal antibody; wt, wild type.
shown that the major proteins in the complex sediment together, indicating that they are components of the same membrane domains (32). At the aggregation stage of development, gp80 is the main component of these complexes, suggesting that they participate in gp80-mediated adhesion. Indeed, large sterol-rich domains have been localized to gp80-mediated cell-cell contacts, and sterol sequestration weakens cell-cell adhesion (31). It is also evident that raftlike domains facilitate gp80 oligomerization and adhesion complex formation (32), but the mechanism of cytoskeleton attachment to these complexes remains to be elucidated. During Dictyostelium aggregation, Tiff does become associated with cytoskeletal complexes and Tiff itself contains a number of cytoskeletal proteins, including actin, comitin, regulatory myosin light chain, and ponticulin (31, 32).

We hypothesized that ponticulin is the primary link between gp80 adhesion complexes and the actin cytoskeleton, since it is a major high affinity actin-binding protein in the plasma membrane of Dictyostelium cells (33). To address this hypothesis and assess how such connections form and function, we monitored interactions among gp80, ponticulin, and actin through subcellular fractionation and localization studies in gene deletion and drug-treated cells during development. We found that gp80 is involved in establishing Tiff-cytoskeleton interactions during development. A major proportion of cellular ponticulin is associated with Tiff and the cytoskeleton and is required to mediate their interaction. gp80 apparently regulates ponticulin function by recruiting it into Tiff and cell-cell contacts. Ponticulin also has an effect on the size of gp80 adhesion complexes within multicellular streams.

EXPERIMENTAL PROCEDURES

Cytoskeleton Interactions with Raftlike Adhesion Complexes

Cell Growth and Development—Dictyostelium discoideum strains were cultured in association with Klebsiella aerogenes (34). In most experiments, the csaA-, gp80-null strain (GT10) (17) was compared with its parental strain AX2, and the ponA-, ponticulin-null strain (TT24.1) (33) was compared with its parental strain KAX3. For development in suspension, cells were collected, washed free of bacteria, and resuspended at 2 × 10^7 cells/ml in 17 mM sodium phosphate buffer (pH 6.1) and then shaken at 180 rpm. Cells were pulsed with CAMP every 7 min at a final concentration of 2 × 10^−8 M beginning at 2 h of development. Alternatively, cells were developed on polyl-lysine-coated coverslips for 12 h as described previously (4).

Pharmacological Studies—Cells were developed in suspension in 17 mM sodium phosphate buffer (pH 6.1). To perturb the actin cytoskeleton or raftlike domains, cells were treated with 5 μM latrunculin B (Sigma), 0.001% (w/v) digitonin (S. B. Penick and Co., New York, NY), or 0.0005% (w/v) filipin (Sigma).

Detergent Extraction and Immunoprecipitation—For detergent extractions, cells were resuspended at 5 × 10^7 cells/ml in cold extraction buffer (40 mM sodium pyrophosphate, 0.1 mg/ml phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM EGTA, 3 mM sodium azide, 10 mM Tris-HCl, pH 7.6). Triton X-100 was added to a final concentration of 0.2% (v/v), and the suspension was shaken at 180 rpm for 1 min at 4 °C. The insoluble material was collected by centrifugation and washed. For Tiff isolation, the resulting pellet was increased to 45% (w/w) sucrose in 1.5 ml, deposited on a 0.5-ml 60% (w/w) cushion in a centrifuge tube, and overlaid with a step gradient of 1 ml of 20% and 2.5 ml of 38% (w/w) sucrose. The gradients were centrifuged at 120,000 × g for 1.5 h at 4 °C using X-100 at 4°C. Triton was collected from the interface between 20 and 38% sucrose. The initial steps of the contact region isolation were similar to the Tiff isolation protocol, and the contact regions were isolated from complexes that sedimented to the interface between 45 and 60% sucrose with centrifugation. The material was washed and then digested in a cytoskeleton depolymerizing solution containing 0.1 mM EDTA, 0.2 mM sodium phosphate buffer, pH 7.6 (35). Then the sample was layered on top of a discontinuous gradient of 2.5 ml of 28% and 2.5 ml of 38% (w/w) sucrose in a centrifuge tube and centrifuged at 120,000 × g for 3 h at 2 °C. The contact regions were collected from the interface between 28 and 38% sucrose. The depolymerized cytoskeleton components, released during the contact region isolation protocol after 1 day of dialysis, were collected as supranant after centrifugation at 14,500 × g for 20 min at 2 °C. The cytoskeleton samples were concentrated using a Centricon YM10 centrifugal filtration device (Millipore Corp., Bedford, MA) for further analysis.

Gel Electrophoresis—Protein samples were separated by SDS-PAGE (36). For immunoblot analysis, bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence kit (Amersham Biosciences). Immunoblots were imaged and quantified using the Fluor-S Max multi-imager system (Bio-Rad).

Protein Identification and Quantification and Lipid Analysis—For protein identification by mass spectrometry, silver-stained gel bands were excised, macerated, reduced, alkylated, and then digested with trypsin as previously described (31). Peptide masses were determined using a PerSeptive Biosystems Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc., Foster City, CA). Peptide masses were submitted to the ProFound search engine (available on the Web Wide Web) for matches. Search parameters were held constant and included all Dictyostelium proteins in the NCBI nonredundant data bases, tolerance for peptide mass error of 1 Da, and no missed cut sites per peptide.

Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce), using bovine serum albumin as a standard. For lipid analysis, membrane samples were subjected to gas-liquid chromatography as described previously (31).

Confocal Fluorescence Staining—Cells were collected from suspension cultures and deposited on poly-l-lysine-coated coverslips for 10 min. Alternately, cells were developed on polyl-lysine-coated for 12 h as described (4). Then samples were fixed, stained, and mounted on slides. For double staining of gp80 and F-actin, the cells were fixed in 3.7% (v/v) formaldehyde for 15 min at room temperature followed by 1% (v/v) formaldehyde in ethanol for 5 min at −20 °C. They were incubated with gp80 mAb and then Alexa 488-phalloidin (Molecular Probes, Inc., Eugene, OR) at 1:1 dilution. For double staining of gp80 and ponticulin, the cells were fixed in 1% (v/v) formaldehyde in methanol for 5 min at −20 °C and then incubated in gp80 mAb and ponticulin polyclonal antibodies (37). For staining of gp80 alone, the cells were fixed in methanol for 5 min at −20 °C. Samples were incubated with Alexa 488/568-conjugated secondary antibodies (Molecular Probes) at 1:300 dilution. Laser-scanning confocal microscopy was performed using a Leica TCS SP confocal microscope equipped with a Leica TCS SP confocal system. Detection was maintained within the range of the gray scale to prevent signal saturation. The images were processed using Adobe Photoshop software (Mountain View, CA).

Cell Adhesion Assays—The maintenance of cell aggregation was assayed by depositing 1 ml of intact aggregates in suspension at −2 × 10^7 cells/ml in 17 mM sodium phosphate buffer (pH 6.1) in a 50-ml conical tube. The samples were then shaken at 180 rpm for 30 min, and samples were taken for cell counting. Singlets, doublets, and triplets were counted as dissociated cells, and the percentage of cell aggregation was calculated relative to the total number of cells present. Assays for cell reassociation were also performed (38). Additionally, cell dissociation was assessed under high shear force by counting dissociated cells following 30 s of fast shaking with a Vortex Genie 2 at setting 8 (31).
RESULTS

Colocalization of gp80 and Actin during Cell-Cell Interactions—To investigate the relationship between gp80 and the actin cytoskeleton during cell aggregation, cells were developed on coverslips for 12 h, fixed, and stained for gp80 and F-actin. At noncontact regions of the cell membrane, gp80 staining was low and displayed a more or less even distribution. However, at both initial and mature cell-cell contact regions, extensive areas of strong gp80 staining were observed (Fig. 1A). For F-actin, strong staining was detected at filopodia emanating from noncontact regions, cellular protrusions mediating initial cell-cell contacts, and smooth membrane interfaces at mature contacts (Fig. 1B). When compared at noncontact regions, no clear relationship was evident between gp80 and F-actin, since only low levels of gp80 were present at the sites with high levels of F-actin (Fig. 1C). In contrast, high levels of gp80 and F-action colocalized in cell-cell contact regions, although their distributions lacked complete overlap, especially at initial cell-cell contacts where F-actin-rich protrusions appeared to extend beyond the established contact (Fig. 1C). Thus, high levels of F-actin alone are not sufficient to organize detectable gp80 complexes at noncontact regions, but rather cell-cell contact is required to instigate their assembly.

gp80 Induces TIFF-Cytoskeleton Interactions during Development—A biochemical approach was taken to address the involvement of raftlike membranes and gp80 in the establishment of cytoskeleton connections to gp80 adhesion complexes. We have shown previously that TIFF co-fractionates with the Triton X-100-insoluble cytoskeleton in a form termed “contact regions” (31). These complexes are rich in sterols and contain many of the proteins found in TIFF. They also have the morphology of stacked adherent membranes and co-fractionate with the cytoskeleton at 12 h of development but not at the single cell stage at 0 h (35). The contact regions can be released from the cytoskeleton as a low density membrane fraction after the depolymerization of the cytoskeleton (Fig. 2A). To characterize the cytoskeletal components, the depolymerized complexes were separated from the membranes by centrifugation and then concentrated. The concentration procedure led to polymerization of the fraction into a gel, consistent with the nature of an actin and myosin mixture. After separation by SDS-PAGE, all silver-stained bands were excised and analyzed by MALDI-TOF mass spectrometry. Actin and components of the myosin II hexamer (myosin II heavy chain, regulatory myosin light chain, and essential myosin light chain) were identified as major species, and components of the Arp 2/3 complex (Arp 3 and p21-Arc) as minor species (Fig. 2B). Components of the tubulin cytoskeleton were not detected. The data indicate that TIFF interacts with an actin-myosin complex in the contact region fraction.

Since the expression of at least three adhesion molecules is induced between 0 and 12 h of development, cells were fractionated at 0, 4, and 8 h to determine which of them contribute to the formation of the detergent-insoluble complexes. TIFF protein displayed a ~2-fold increase over the time period (Fig. 2C). For the contact regions, however, only background levels of protein were recovered up to 4 h, a stage at which the EDTA-sensitive adhesion molecules mediate aggregation. At
8 h, recovery of contact regions increased by 10-fold (Fig. 2D).
Since high levels of gp80 were expressed by 8 h, we examined the role of gp80 in the interaction between TIFF and the cytoskeleton by comparing wild-type and gp80-null (csaA−) cells at 12 h of development. The level of TIFF protein was reduced by ~50% in gp80-null cells relative to wild-type cells (Fig. 2E). However, protein recovery in the contact region fraction was reduced to background levels in gp80-null cells (Fig. 2F). Since stable contact regions could only be recovered with the expression of gp80, they probably represent gp80 adhesion complexes.

The lower levels of TIFF protein detected in the gp80-null cells could be due partly to the physical absence of gp80, since it is the major protein component of TIFF (31). Indeed, lipid analyses revealed that similar levels of TIFF lipids were recovered from the wild-type and gp80-null (csaA−) cells at 12 h. However, protein recovery in the contact region fraction was reduced to background levels in gp80-null cells (Fig. 2F). Since stable contact regions could only be recovered with the expression of gp80, they probably represent gp80 adhesion complexes.

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To test whether ponticulin mediates the interactions between the gp80 complexes and the actin cytoskeleton, we analyzed wild-type and ponticulin-null (ponA−) cells (Fig. 3B). In wild-type cells, gp80 and actin displayed distribution patterns similar to the controls described above (see Fig. 3A). For ponticulin-null cells, however, gp80 was lost from TIFF and became predominantly found in TISF, similar to the results of latrunculin B treatment of wild-type cells. Analyses of actin revealed its complete loss from the TISF of null cells, suggesting that ponticulin was required for the binding of actin to TIFF. A partial loss of actin was also observed in TIFF, but to
a lesser extent than that resulting from the latrunculin B treatment, suggesting that the actin cytoskeleton remained largely intact. Taken together, these results suggest that ponticulin is required for the gp80 adhesion complex to establish cytoskeleton connections.

Ponticulin Associates Primarily with TIFF and the Cytoskeleton—Next, we assessed the proportion of total cellular ponticulin that may be involved specifically in the TIFF-cytoskeleton interactions. Cell aggregates were collected at 10 h of development, extracted with cold 0.2% Triton X-100, and fractionated into detergent-soluble supernatants and detergent-insoluble pellets. SDS-PAGE and protein blot analyses were performed to assess the partitioning of ponticulin between the two fractions (Fig. 4). To address the bases for detergent insolubility, we pretreated the cells with either latrunculin B, which induces actin depolymerization, or low levels of digitonin and filipin. Digitonin was used in this study to maintain cell structure, and filipin, which perturb rafts by sequestering membrane sterols. In carrier controls, ~75% of total cellular ponticulin partitioned into the detergent-insoluble pellets (Fig. 4). gp80 displayed a slightly greater degree of solubilization, consistent with our previous observation (31). With latrunculin B pretreatment, ponticulin displayed enhanced solubilization, resulting in even partitioning between the pellet and supernatant fractions (Fig. 4). After digitonin or filipin treatment, the detergent solubility of ponticulin increased to ~50% and ~40%, respectively (Fig. 4). For digitonin or filipin treatment, the detergent solubility of ponticulin increased to ~50% and ~40%, respectively, suggestive of additive effects (Fig. 4). Thus, the insolubility of ponticulin in Triton X-100 is probably due to its association with both TIFF and the actin cytoskeleton.

In contrast to ponticulin, gp80 was not significantly affected by the drug treatments (Fig. 4). Low levels of filipin and digitonin were used in this study to maintain cell structure, and they were 10-fold lower than those required to block TIFF recovery (27). In comparison with other TIFF components, ponticulin appears to be more sensitive to the sequestration of membrane sterols.

Lack of Direct Interactions between Ponticulin and gp80—We have shown previously that gp80 and ponticulin are components of the same raftlike domain (32). The above results also indicate that ponticulin is a major link between the gp80 adhesion complexes and the cytoskeleton, but do these proteins interact directly? Since substantial amounts of both ponticulin and gp80 can be solubilized under fairly gentle conditions of 0.2% cold Triton X-100 after treatment with latrunculin B or the sterol sequestering agents, we determined whether these proteins could be co-immunoprecipitated from the resulting supernatants (Fig. 5). Under six different conditions, including those with drug pretreatments and the untreated controls, ~50% of gp80 could be specifically immunoprecipitated. However, ponticulin remained in the unbound fraction in all of the conditions. Thus, direct gp80-ponticulin interactions were not apparent.

gp80 and Membrane Sterols Act Together to Recruit Ponticulin into TIFF—Since direct interactions between gp80 and ponticulin could not be detected in Triton X-100-soluble complexes, we assessed whether membrane sterols played a role in the co-fractionation of gp80 and ponticulin in TIFF and TISF. Pretreatment with low levels of digitonin or filipin did not affect the partitioning of gp80 into TIFF (Fig. 6A). In contrast, the amount of ponticulin present in TIFF was markedly reduced, suggesting that its co-fractionation with gp80 was dependent upon membrane sterols. However, ponticulin associated with TISF were not affected (Fig. 6A), suggesting that fully assembled adhesion complexes might resist the sterol sequestration.

The role of gp80 in the partitioning of ponticulin into TIFF and TISF was examined using wild-type and gp80-null cells. In the gp80-null cells, the level of ponticulin in TIFF was markedly reduced, whereas its level in TISF remained relatively high (Fig. 6B). These results were similar to those obtained by sequestering membrane sterols, suggesting that gp80 may act through sterols to recruit ponticulin into raftlike domains in the plasma membrane.

Requirement of gp80 for Ponticulin Recruitment to Cell-Cell Contacts—To determine whether gp80 acts upstream of ponticulin during the assembly of adhesion complexes, the subcellular distributions of gp80 and ponticulin were examined by confocal microscopy in wild-type and mutant cell aggregates after 10 h of development in suspension. In wild-type cell aggregates, high levels of gp80 and ponticulin colocalized at cell-cell contacts, and both displayed lower staining intensity at noncontact regions (Fig. 7). In ponticulin-null aggregates, strong gp80 staining was also detected along cell-cell contacts, suggesting that gp80 was able to assemble into large adhesion complexes in the absence of ponticulin. In gp80-null cells, the ponticulin antibodies detected low levels of even staining along the plasma membrane and some punctate cytoplasmic staining. However, ponticulin enrichment at cell-cell contacts was not detected (Fig. 7). Since gp80 can assemble into cell-cell contacts in the absence of ponticulin and is required to recruit ponticulin to contact regions, gp80 appears to act upstream of ponticulin during the assembly of adhesion complexes.

Effects of Latrunculin B on gp80-Mediated Adhesion in Wild-type and Mutant Strains—To investigate the relationship between gp80-mediated cell-cell adhesion and the cytoskeleton, 10-h cell aggregates were exposed to latrunculin B and then monitored for dissociation (Fig. 8A). Wild-type cell aggregates were able to withstand the latrunculin B treatment, and ~85% of cells remained in aggregates in both treated and control samples. gp80-null cells dissociated readily with the latrunculin B treatment that reduced their aggregation from 70 to 25%. However, ponticulin-null cell aggregates were able to withstand the latrunculin B treatment and exhibited minimal dissociation in both treated and control cells, similar to wild-type cells. Ponticulin-null cells and latrunculin B-treated cells were also subjected to two additional adhesion assays: a cell dissociation assay to test the degree aggregate dissociation under shear forces and a cell reassociation assay to test the reaggregation of dissociated cells. However, no differences in gp80-mediated adhesion were observed relative to controls (data not shown). These results show that gp80 is unique among the adhesion systems expressed in early development, since it can mediate cell-cell adhesion despite F-actin perturbation or ablation of its principle link to the cytoskeleton.

When cell aggregates were stained with phalloidin to examine the actin cytoskeleton of the wild-type and mutant strains, the overall patterns of F-actin staining were indistinguishable between the strains and resembled the data in Fig. 1B (data not shown). Since subtle differences might be present, we treated the cells with latrunculin B and examined the cytoskeleton remnants, reasoning that they might represent stable actin complexes. In wild-type aggregates, small foci of F-actin...
were observed, distributed more or less evenly along cell-cell contacts (Fig. 8B). gp80 was also detected along the contacts but with a more smooth and continuous distribution (Fig. 8C).

However, in gp80-null aggregates, the latrunculin B treatment resulted in a lower degree of F-actin staining at cell-cell contacts, and a smaller number of strongly stained structures were detected at random positions in the aggregates (Fig. 8D). In ponticulin-null cell aggregates, residual F-actin was distributed along the cell-cell contacts but in a more diffuse and continuous pattern relative to wild-type (Fig. 8E). It is, therefore, possible that gp80 and ponticulin are involved in remodeling cytoskeleton structure at cell-cell contact regions.

gp80 Adhesion Complexes Are Elongated in Streams of Ponticulin-null Cells—Although ponticulin was apparently dispensable for gp80-mediated cell-cell adhesion during development in suspension, ponticulin may have effects on gp80 adhesion complexes during more dynamic cell-cell interactions. Thus, we examined the distribution of gp80 in wild-type and ponticulin-null cells at the streaming stage of development. In wild-type cell streams, gp80 was often enriched at end-to-end contacts in C-shaped staining patterns (Fig. 9A). In ponticulin-null cells, gp80 staining appeared to be more evenly distributed along and around the cells (Fig. 9B). To quantify the length distribution of the gp80 staining patterns, the tonal levels in the confocal images were converted automatically into white, gray, or black (Fig. 9, C and D). Measurement and statistical analysis of the length distributions revealed a significant difference between the wild-type and ponticulin-null cells. In wild-type streams, the average complex length measured was 5.1 μm, and only 35% of the complexes extended past 5 μm (Fig. 9E). In ponticulin-null streams, the average complex length was 6.4 μm, and 53% of the complexes extended past 5 μm (Fig. 9F). Thus, gp80 adhesion complexes tended to be longer in the ponticulin-null cells.

**DISCUSSION**

During Dictyostelium development, strong cell-cell adhesion is mediated by a gp80 adhesion complex composed of a core of...
oligomerized receptors integrated into opposing raftlike domains (31, 32). In this paper, we have demonstrated that specific cytoskeleton connections are established with the complex via ponticulin, a major mediator of interactions between the actin cytoskeleton and the plasma membrane in Dictyostelium (33). Our results frame a model for complex assembly and provide insights into how the complex may function during morphogenesis.

In contrast to transmembrane adhesion molecules that interact directly with cytoskeleton adapter proteins, gp80 appears to interact indirectly with ponticulin. Ponticulin is an atypical membrane protein containing transmembrane domains and a phospholipid anchor (39). Its extracellular loops are predicted to be short and close to the membrane, and its cytoplasmic loops bind to the sides of actin filaments with high affinity. The region of gp80 in proximity to the membrane is predicted to form a linear stalk that extends perpendicularly away from the membrane (40). If interactions occur between the short loops of ponticulin and the linear stalk of gp80, they are probably weak, since we were unable to co-immunoprecipitate the proteins after mild detergent solubilization (Fig. 5).

![Fig. 5. Ponticulin does not co-immunoprecipitate with gp80. To examine whether gp80 and ponticulin have direct protein-protein interactions, immunoprecipitation of gp80 was carried out following solubilization of 10 h cells with cold 0.2% Triton X-100. Insoluble complexes were removed by centrifugation, and gp80 mAb was added to the supernatants. Protein A-Sepharose beads were then added, and equal proportions of unbound material (U) and bound material (B) were separated by 12% SDS-PAGE, blotted on nitrocellulose, and probed for gp80 and ponticulin. The procedure was performed in the absence of gp80 mAb to control for nonspecific binding to the beads. The experiments were performed with untreated cells and after pretreatment with 5 μM latrunculin B alone, 0.001% (w/v) digitonin alone, 0.0005% (w/v) filipin alone, 0.001% (w/v) digitonin plus 5 μM latrunculin B, or 0.0005% (w/v) filipin plus 5 μM latrunculin B. In each case, ~50% of detergent-solubilized gp80 was immunoprecipitated in the bound fraction. Ponticulin remained entirely in the unbound fractions.](http://www.jbc.org/)

| Untreated | Lat B | Digitonin |
|-----------|-------|-----------|
| gp80 mAb  | +     | -         | +         |
| Ponticulin| +     | -         | +         |

Fig. 6. Ponticulin displays reduced partitioning into TIFF after sterol sequestration and also in gp80-null cells. A, to assess whether membrane sterols are involved in mediating interactions between gp80 and ponticulin, 10-h wild-type cells were treated with 0.001% (w/v) digitonin, 0.0005% (w/v) filipin, or Me2SO carrier and fractionated into TIFF and TISF using single discontinuous gradients. One-ml fractions were collected, and equivalent volumes were separated by 12% SDS-PAGE, blotted, and probed for gp80, ponticulin, and actin. Sterol sequestration resulted in a loss of ponticulin from TIFF, whereas the pattern for gp80 remained unchanged. The results are representative of two experiments. B, to examine the effects of gp80 on the partitioning of ponticulin, samples of TIFF and TISF were prepared from equivalent numbers of wild-type and csaA cells at 10 h of development and examined as above. The results are representative of three separate experiments.
actions prior to cell-cell contact, since larger complexes were not detected by light microscopy prior to cell aggregation (Fig. 1). By analyzing TIFF, we have found that a basal level of actin is associated with raftlike membranes through ponticulin, even without the expression of gp80 (Fig. 6B). However, gp80 oligomers are insensitive to latrunculin B, and their assembly may not necessarily depend on the cytoskeleton interaction (32).

In contrast to many adhesion complexes that require participation of the actin cytoskeleton for their assembly (11), nascent gp80 adhesion complexes appear to assemble with minimal cytoskeleton association. As cells come into contact, the trans-homophilic interaction of gp80 has the potential to cross-link the gp80 cis-oligomers into large adhesion complexes, and we have proposed that associated rafts are cross-linked and coalesced into enlarged raftlike domains as a result (20, 32). Indeed, gp80 is able to assemble functional adhesion complexes at cell-cell contacts of ponticulin-null cells, despite their lack of cytoskeleton association. Consistent with this observation, model membrane studies have demonstrated that gp80-gp80 interactions alone can produce extensive expansion of intermembrane contacts (42). Furthermore, large sterol-rich domains have been found at sites of gp80-mediated cell-cell adhesion (20), as they have been observed at bacterial attachment sites to macrophages (43) and mast cells (44). Large domains enriched in raft lipids have also been reported for cell-cell contacts among immune cells (45).

The formation of the nascent gp80 adhesion complexes may

![gp80](image1)
![ponticulin](image2)

**Fig. 7. gp80 recruits ponticulin to cell-cell contacts.** Wild-type and mutant cells were developed in suspension for 10 h and then processed for confocal microscopy. Top panels, the distributions of gp80 and ponticulin are compared in a wild-type aggregate. Bottom left, the distribution of gp80 in a ponA− cell aggregate is shown. Bottom right, the distribution of ponticulin in a csaA− cell aggregate is shown. Bars, 5 μm.
be critical for the recruitment of ponticulin. In the formation of large raftlike domains at cell-cell contacts, gp80 could relocalize the pool of ponticulin normally partitioned into rafts found throughout the plasma membrane. Indeed, gp80 is required for the accumulation of ponticulin at cell-cell contacts (Fig. 7). Additionally, gp80 may promote the recruitment of ponticulin into rafts. This effect is evident from the markedly reduced level of ponticulin in TIFF isolated from gp80-null cells (Fig. 6B). Since cross-linking and coalescence of rafts at the nascent adhesion complexes would dramatically decrease the ratio between the circumference and the interior area of the domains, there might be fewer chances for ponticulin to transfer to the nonraft part of the membrane, and its partitioning into the expanded raftlike domains could increase. This effect may be particularly important for the recruitment of ponticulin, since its association with TIFF is more sensitive to sterol sequestration than gp80 and other TIFF components.

Ponticulin is required for the assembly of stable gp80 adhe-

![Image](image-url)
Cytoskeleton Interactions with Raftlike Adhesion Complexes

complexes that co-fractionate with the actin-myoosin cytoskeleton. Ponticulin is also known to assemble extensive networks of actin filaments in close apposition with the plasma membrane at the basal cell surface (33). Thus, ponticulin may organize similar actin networks at gp80 complexes. In fact, a large proportion of ponticulin is apparently involved in TIFF-raft protein comitin that mediates interactions between Golgi cytoskeletons in mammalian cells (47, 48). Additionally, the activity with specific but unidentified lipids (46). Certain lipids cytoskeleton interactions, and ponticulin-based changes to cytoskeleton. Ponticulin is also known to assemble extensive large proportion of ponticulin is apparently involved in TIFF-organize similar actin networks at gp80 complexes. In fact, a membrane at the basal cell surface (33). Thus, ponticulin may interact with latrunculin B, it may also be unaffected by natural gp80 interactions in epithelial cells (55) and in muscle cells (56). Ponte, E., Bracco, E., Faix, J., and Bozzaro, S. (1998) Cell. Mol. Life Sci. 55, 359–367. Viola, A., Schroeder, S., Sakakibara, Y., and Lanzavecchia, A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9360–9365. Simons, K., and Toomre, D. (2000) Mol. Membr. Biol. 17, 183–198. Rothenberg, M. E., and Leder, P. (1990) Cell 62, 1235–1246. Provided by guest on July 25, 2018http://www.jbc.org/ Downloaded from
Cytoskeleton Interactions Involved in the Assembly and Function of Glycoprotein-80 Adhesion Complexes in *Dictyostelium*

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