Assembly of Glycoprotein-80 Adhesion Complexes in Dictyostelium

RECEPTOR COMPARTMENTALIZATION AND OLIGOMERIZATION IN MEMBRANE RAFTS

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The phospholipid-anchored membrane glycoprotein (gp)-80 mediates cell-cell adhesion through a homophilic trans-interaction mechanism during Dictyostelium development and is enriched in a Triton X-100-insoluble floating fraction. To elucidate how gp80 adhesion complexes assemble in the plasma membrane, gp80-gp80 and gp80-raft interactions were investigated. A low density raft-like membrane fraction was isolated using a detergent-free method. It was enriched in sterols, the phospholipid-anchored proteins gp80, gp138, and pонтiculun, as well as DdCD36 and actin, corresponding to components found in the Triton X-100-insoluble floating fraction. Chemical cross-linking revealed that gp80 oligomers were enriched in the raft-like membrane fraction, implicating stable oligomer-raft interactions. However, gp80 oligomers resisted sterol sequestration and were partially dissociated with Triton X-100, suggesting that compartmentalization in rafts was not solely responsible for their formation. The trans-dimer known to mediate adhesion was identified, but cis-oligomerization predominated and displayed greater accumulation during development. In fact, oligomerization was dependent on the level of gp80 expression and occurred among isolated gp80 extracellular domains, indicating that it was mediated by direct gp80-gp80 interactions. Rafts existed in gp80-null cells and such pre-existing membrane domains may provide optimal microenvironments for gp80 cis-oligomerization and the assembly of adhesion complexes.

Integral membrane proteins and lipids have been predicted to typically display long-range random distributions, and maintenance of any nonrandom distributions would require specific mechanisms (1). In recent years, many nonrandom distributions have been discovered. For individual proteins, long-range lateral diffusion can be restricted by corals established by the underlying cytoskeleton (2). The cytoskeleton can also tether and actively redistribute transmembrane proteins (3). Discrete membrane structures, such as cell-cell and cell-substratum adhesion complexes, also form through interactions between multiple membrane components (4). Modulation of the structure and function of these complexes may influence many cellular and developmental events.

Glycosylphosphatidylinositol (GPI)-anchored proteins are unique, since they lack the direct cytoskeleton interactions that affect the distributions and complexes of their transmembrane counterparts. Nevertheless, they can be clustered into rafts by lipid interactions (5, 6). Rafts are membrane microdomains that form from the close packing of sterols and saturated lipids into a liquid ordered structure. Model membranes with this structure are insoluble in cold Triton X-100. Triton X-100-insoluble floating fractions (TIFF) have been isolated from many different cell types and contain the lipid components of rafts, plus GPI-anchored and other acylated proteins (7–10). A growing number of GPI-anchored cell adhesion molecules have been discovered in TIFF (11–14), suggesting that they are components of membrane rafts. This potential compartmentalization could possibly effect receptor interactions, cytoskeleton associations, and signaling during the assembly of GPI-anchored cell adhesion molecule complexes.

We have used Dictyostelium as a model system to examine the involvement of TIFF in cell-cell adhesion mediated by GPI-anchored receptors (14). Dictyostelium has a simple and well defined life cycle that permits the biochemical analysis of dynamic processes over developmental time (15). During the aggregation stage of development, single cells aggregate chemotactically and undergo intercellular adhesion to form stable aggregates (16). Cell-cell contacts are formed first by the Ca$^{2+}$ dependent adhesion molecule DdCAD-1 (17, 18) and distinct Mg$^{2+}$-dependent adhesion sites (19). Subsequently, two Ca$^{2+}$/Mg$^{2+}$-independent adhesion molecules, gp80 and gp150/LagC, are expressed (20–22).

Gp80 is a phospholipid-anchored receptor (23, 24) which mediates cell-cell adhesion through a trans-homophilic binding mechanism (25). Gp80 is required for strong adhesion that maintains multicellularity during development (26, 27). However, gp80 adhesion complexes must also be dynamic structures, since cells are constantly breaking and re-making contacts as they migrate. What mechanisms underlie the assembly and disassembly of gp80 adhesion complexes?

Like many vertebrate GPI-anchored adhesion molecules (11–13), gp80 is a main component of TIFF (14). Additionally, Triton X-100-insoluble contact regions (28) were found to be a cytoskeleton-associated form of TIFF and large domains containing TIFF proteins and lipids localize to gp80-mediated...
cell-cell contacts, thus implicating a role for TIFF in gp80-mediated adhesion. In this paper, we have extended our studies to investigate the assembly and organization of gp80 molecules in raft-like membrane domains as gp80-mediated cell-cell contacts form during development. Low density plasma membrane fragments were isolated using a detergent-free method. Their similarity to TIFF confirms the existence of raft-like domains in the Dictyostelium plasma membrane. Gp80 was found to oligomerize preferentially in these domains by direct cis-interactions between their extracellular domains. These results suggest that raft-like domains may provide optimal microenvironments for the assembly of gp80 cis-oligomers and their subsequent incorporation into adhesion complexes.

**Experimental Procedures**

Cell Growth and Development—Both the wild-type axenic strain AX2 and the csaA-null strain GT10 (26) of Dictyostelium discoideum, were cultured with Klebsiella aerogenes (29). For development, cells were collected, washed, and resuspended at 2 × 10^7 cells/ml in 17 mM sodium phosphate buffer (pH 6.1), shaken at 180 rpm and pulsed with cAMP every 7 min at a final concentration of 2 × 10⁻⁴ M.

Isolation of TIFF and Low Density Plasma Membrane Fragments—TIFF was isolated as previously described (14). Low density plasma membrane fragments were isolated according to an established protocol (30). Cell pellets were frozen, thawed, resuspended at 1.5 × 10⁶⁰ cells/ml in 1 mM ZnCl₂, and homogenized using a Dounce homogenizer. Microscopic examination revealed minimal breakage of nuclei. Cell particulates were collected by centrifugation at 3,000 × g and plasma membranes were isolated using the aequous two-phase polymer system (31). After washing, isolated plasma membranes were resuspended in 8.5% (w/v) sucrose, 1 mM EDTA, 2 mM phosphate buffer (pH 7.6), and sonicated with 1210 s pulses of a Sonifer cell disruptor (Branson, Danbury, CT) with a duty cycle of 40% and an output of 4, on ice. The suspension was mixed with 60% (w/v) sucrose in 20 mM phosphate buffer (pH 7.6) for a final sucrose concentration of ~5% (w/w). This mixture was overlaid with either a continuous (30–45%, w/w) or a discontinuous (20/38%, w/w) sucrose gradient in 20 mM sodium phosphate buffer (pH 7.6) and centrifuged at 120,000 × g for 15–17 h at 2 °C, using the Beckman SW40 rotor. One-ml fractions were collected from the top for further analyses.

Immunoprecipitation of Membranes—Equivalent amounts of isolated low density plasma membrane fragments were incubated with antibodies as previously described (14). Samples were incubated with 0.1 mg/ml anti-gp80 IgG or 0.1 mg/ml of an irrelevant mouse IgG in TBS plus 0.1% bovine serum albumin. After washing, samples were incubated with goat anti-mouse antibodies conjugated with 10-nm gold (Sigma) at 1:10 dilution in TBS plus 0.1% bovine serum albumin. After washing, pellets were fixed in 40% (v/v) acetone and 60% (w/v) sucrose phosphate buffer (pH 7.6) and then raised to ~57% (w/w) sucrose, overlaid with a continuous gradient of 34–45% (w/v) sucrose and centrifuged at 120,000 × g for 15–17 h at 2 °C. One-ml fractions were collected from the top of the gradient and proteins were collected by chloroform-methanol precipitation.

Preparation of Gp80 Extracellular Domains—The extracellular domains of gp80 were isolated from culture medium using a reported method (23). Cells were developed in suspension for 10 h and then pelleted at 30,000 × g. The supernatant was centrifuged at 100,000 × g for 1 h at 4 °C. The resulting S-100 supernatant was adjusted to pH 7.6 and then concentrated using the Centricon centrifugal filtration device (Millipore, Bedford, MA).

Confocal Microscopy—Samples were cross-linked with DSP, DSS, or BS3 (Pierce, Rockford, IL). In a typical experiment, samples of equal protein concentration were incubated with 0.1 mM cross-linker in 20 mM sodium phosphate buffer (pH 7.6) for 30 min at room temperature. Unreacted cross-linker was quenched by the addition of 1 mM Tris-HCl (pH 7.6) to a final concentration of 100 mM for 10 min. Cells were cross-linked at 2 × 10⁷ cells/ml in 17 mM sodium phosphate buffer (pH 7.6) with rotation at 80 rpm.

Gel Electrophoresis—Protein concentrations were determined using the bichinchoninic acid protein assay kit (Pierce). Protein samples were separated by SDS-PAGE (32). For immunoblot analysis, bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence kit (Amersham Bioscience, Inc., Fiscatway, NJ). Immunoblots were imaged and quantified using the Fluor-S Max multimager system (Bio-Rad, Mississauga, ON). Two-dimensional SDS-PAGE was performed by first separating samples in a nonreducing 6% acrylamide slab gel. Separation in the second dimension was carried out in a 10% slab gel. Before the second dimension, stacking gel buffer containing 10% β-mercaptoethanol was applied to the stacking gel for 15 min and then removed. The lane from the first gel was excised, placed on top of the stacking gel of the second gel, and encased in molten 1% (w/v) agarose, 2% β-mercaptoethanol in stacking gel buffer.

Protein Identification 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 (14). MALDI-TOF mass spectrometry was performed at the Mass Spectrometry Laboratory of the Molecular Medicine Research Center, University of Toronto. Monoisotopic peptide masses were submitted to the ProFound search engine (available on the world wide web) for matches. Unless otherwise stated, search parameters were held constant and included all Dictyostelium proteins in the NCBI nonredundant or SWISS-PROT data bases, tolerance for peptide mass error of 300 ppm, and no missed cut sites per peptide. For lipid analysis, membrane samples were subjected to gas-liquid chromatography as described previously (14).

Immunofluorescent Staining—Cells were collected from suspension cultures and deposited on poly-L-lysine-coated coverslips for 10 min. Membrane samples were deposited on coated coverslips for 20–45 min. The samples were then fixed, stained, and mounted on slides as previously described (33). Samples were incubated with antibodies against gp80 followed by Alexa 488-conjugated secondary antibodies (Molecular Probes, Eugene, OR) at 1:300 dilution. Laser scanning confocal microscopy was performed using a Leica DM IRBE inverted microscope equipped with a Leica TCS SP confocal system. Detection was maintained within the range of the gray scale to prevent signal saturation. Capping of gp80 was carried out using the anti-gp80 mAb 80L5C4 in the absence of secondary antibody using an established protocol (14). Cell aggregates were dispersed and deposited on coverslips for 10 min. Subsequently, cells were incubated with the mAb for 30 min, and then washed and incubated until 55 min after the initial addition of the mAb. The cells were then fixed and prepared for confocal microscopy.

Results

Isolation of Low Density Plasma Membrane Fragments Enriched in Gp80—The phospholipid-anchored cell adhesion molecule gp80 is enriched in TIFF (14). To demonstrate that TIFF originated from discrete raft-like domains in the plasma membrane, a detergent-free isolation method was developed based on the one used for caveolae isolation (30). Plasma membranes were purified, sonicated, and subjected to flotation centrifugation into a continuous sucrose gradient. Without prior sonication, the plasma membrane preparation formed a single prominent band at a density of 1.19 g/ml. The band was collected in fraction 6 (termed fraction 6 hereafter) (Fig. 1A), which contained gp80 and most of the membrane protein. Proteins were not detected in the lower density fractions. With prior sonication, the main band shifted to a higher density region at 1.20 g/ml and was collected in fraction 7 (designated fraction s7 hereafter) (Fig. 1B). Additionally, proteins of 139, 105, 82, 41, and 16 kDa floated into the lower density fractions with maximum levels detected in fraction 4 (termed fraction s4 hereafter). Fraction s4 had a density of 1.17 g/ml, indicating the association of these proteins with lipid-rich domains of the plasma membrane. Relative to total protein, gp80 was enriched by 3–5-fold in fraction s4 over fraction s7.

The association of gp80 with these fractions was also examined by confocal microscopy. Fixed membrane samples were stained for gp80. The nonsonicated membranes in fraction 6 displayed a heterogeneous gp80 distribution, whereas gp80-rich regions of <0.5 μm in diameter were embedded in the bulk of the large membrane fragments (Fig. 1C). Fraction s4 contained an abundance of gp80-stained small membrane fragments (Fig. 1D), which might represent the gp80-rich regions observed in fraction 6.

Protein and Lipid Components of the Low Density Plasma Membrane Fragments—Next, we analyzed the protein and lipid composition of the gp80-enriched low density membrane fractions. The protein components of the low density membrane fractions were isolated and analyzed by 2D gel electrophoresis and mass spectrometry. MALDI-TOF mass spectrometry was used to identify proteins present in the low density membrane fractions. The MALDI-TOF mass spectrometry results were cross-checked with the Mass Spectrometry Laboratory of the Molecular Medicine Research Center, University of Toronto. The results of the MALDI-TOF mass spectrometry analysis of the low density membrane fractions are shown in Table 1. The low density membrane fractions contained a variety of proteins, including several members of the lipid raft-associated protein family. These proteins were identified using MALDI-TOF mass spectrometry and confirmed using 2D gel electrophoresis and mass spectrometry. The results of the 2D gel electrophoresis and mass spectrometry analysis of the low density membrane fractions are shown in Figure 2. The 2D gel electrophoresis results showed that the low density membrane fractions contained a variety of proteins, including several members of the lipid raft-associated protein family. These proteins were identified using MALDI-TOF mass spectrometry and confirmed using 2D gel electrophoresis and mass spectrometry. The results of the 2D gel electrophoresis and mass spectrometry analysis of the low density membrane fractions are shown in Table 1.
lipid compositions of fraction s4. Fifteen bands were excised from a silver-stained gel and then analyzed by MALDI-TOF mass spectrometry. Eight produced relatively strong mass spectra, and six of them were identified as the *Dictyostelium* proteins gp138, gp138B, DdCD36, gp80, actin, and ponticulin (Fig. 1E). The identifications of gp80 and actin were confirmed by immunoblot analysis. Post-source decay analysis was used to confirm the other identifications, and 18/20, 9/10, 19/21, and 49/54 of derived peptide fragments matched (error <1 Da) expected fragments of peptides 326–338 of gp138, 484–495 of gp138B, 635–656 of DdCD36, and 66–89 of ponticulin, respectively. All the proteins identified here have been found in TIFF (14).

Total lipids were extracted and quantified (Table I). Lipid-to-protein ratios were highest for the low density plasma membranes fragments and lowest for fraction s7, consistent with their apparent densities in the sucrose gradient. The phospholipid and sterol contents were analyzed. Relative to fraction 6, the sterol-to-phospholipid ratio of fraction s7 was reduced by ~40%, whereas fraction s4 showed an increase of ~36%. A 2-fold higher sterol-to-phospholipid ratio was observed in fraction s4 relative to fraction s7. The protein composition of fraction s4 and its high sterol level indicated that the low density membrane fragments had raft-like properties.

**Recovery of Raft-like Membrane Fragments from gp80-null Cells**—To investigate the role of membrane rafts in the assem-

![Image](https://via.placeholder.com/150)
ably of gp80 adhesion complexes, we first determined whether the raft-like membrane domains required gp80 for their formation. Plasma membranes were isolated from csaA-null cells where the gene encoding gp80 was inactivated (26). Membrane samples were subjected to flotation into a continuous sucrose gradient with or without prior sonication (Fig. 2). The protein profiles of the gradient fractions were similar to those obtained with wild-type cells (see Fig. 1). With sonication, the bulk of the membrane moved to fraction 7 from fraction 6 where it banded without sonication. Although gp80 was absent, proteins corresponding to the gp138 species, DdCD36, actin, and ponticulin floated into fractions 3 and 4 only after sonication. Total lipids were extracted and quantified from these membrane fractions. Similar to the samples isolated from wild-type cells, both the lipid-to-protein and sterol-to-phospholipid ratios were highest for the low density plasma membrane fragments, intermediate for fraction 6, and lowest for fraction s7 (Table I). These results thus indicate that raft-like domains exist in the absence of gp80.

Co-fractionation of the Low Density Membrane Components with Gp80—Since the low density membrane fraction might contain several types of raft-like domains, we assessed the relationship between the major protein species in the gp80-enriched raft-like structures using a variation of the co-immunoprecipitation approach. The low density membrane fragments were incubated with either anti-gp80 mAb alone or mAb followed by colloidal gold-conjugated goat anti-mouse antibodies. Samples were floated into a 34–45% (w/w) sucrose gradient to separate membrane fragments that contained immune complexes of gp80 from fragments devoid of gp80. The binding of colloidal gold was expected to shift the gp80-rich fragments to a higher density region. Anti-gp80 antibodies alone shifted these bands further in a region between fractions 5 to 13 (Fig. 3). This wide spread probably resulted from the formation of large immune complexes. Bands corresponding to the gp138 species, DdCD36, gp80, and ponticulin displayed a similar distribution pattern in all cases. Bands between 45 and 70 kDa were detected mainly in fraction 13 and were likely due to bovine serum albumin and protein aggregates in the antibody solutions. Neither bovine serum albumin nor control mAb produced the density shifts. The co-shifting of the major protein species with gp80 is consistent with them being directly or indirectly associated with gp80 in the same membrane rafts.

TABLE I

Lipid compositions of plasma membrane fractions

| Sterols | Phospholipids | Sterols:phospholipids |
|---------|---------------|-----------------------|
| AX2     |               |                       |
| Fraction 6 (0.613) | 0.383 ± 0.205 (1.03) | 0.770 ± 0.164 (0.621) |
| Fraction s7 (0.613) | 0.383 ± 0.205 (1.03) | 0.770 ± 0.164 (0.621) |
| LDPMF (7.21) | 2.04 ± 0.385 (5.48) | 1.69 ± 0.411 (1.36) |
| GT10    |               |                       |
| Fraction 6 (0.613) | 0.383 ± 0.205 (1.03) | 0.770 ± 0.164 (0.621) |
| Fraction s7 (0.613) | 0.383 ± 0.205 (1.03) | 0.770 ± 0.164 (0.621) |
| LDPMF (7.21) | 2.04 ± 0.385 (5.48) | 1.69 ± 0.411 (1.36) |

FIG. 2. Isolation of raft-like membrane fragments from csaA-null cells. Plasma membranes preparations from 10-h csaA-null cells were floated into 30–45% (w/w) sucrose gradients. One-mL fractions were collected from the top. Nonsonicated membranes formed a sharp band collected in fraction 6. Sonicated membranes formed a sharp band collected in fraction 7. Equal proportions of the fractions were separated in a 10% gel. Silver staining revealed protein species with molecular weights of 139,000, 105,000, 41,000, and 16,000, corresponding to the gp138 family members, DdCD36, actin, and ponticulin, respectively, that floated into fractions 5–6 (arrowheads).
The specificity of the chemical cross-linking reaction was assessed by incubating 10-h cells with 0.01–1.0 mM DSS (Fig. 5B). Over this concentration range, the proportion of monomeric gp80 decreased substantially, with a corresponding increase in the level of the cross-linked species. From 0.03–1.0 mM DSS, large gp80 complexes were observed in the stacking
gel. Quantitative analysis showed only a minor reduction in the amount of monomeric gp80 between 0.1 and 1.0 mM DSS. The small change in gp80 cross-linking in response to a 10-fold increase in cross-linker concentration suggested that nonspecific cross-linking was minimal. When cells were cross-linked with 0.01–1 mM DSS for 30 min and probed for gp80, dose effects of cross-linking by DSS. Cells were cross-linked with 0.01–1 mM DSS for 30 min and probed for gp80 oligomers. Accumulation of high molecular weight oligomers was apparent in the stacking gel. C, time course of cross-linking by 0.1 mM DSS. In both B and C, the relative amounts of monomeric gp80 were quantified and plotted below the Western blots. Values are normalized to those of the noncross-linked samples and represent the mean of three experiments.

**FIG. 5. Analyses of gp80 oligomers on live cells.** A, cells at 10 h of development were cross-linked with 0.1 mM DSS or 0.1 mM BS3 for 30 min at 2 x 10⁷ cells/ml. Samples were separated on a 5% gel, blotted, and probed for gp80. B, dose effects of cross-linking by DSS. Cells were cross-linked with 0.01–1 mM DSS for 30 min and probed for gp80 oligomers. Accumulation of high molecular weight oligomers was apparent in the stacking gel. C, time course of cross-linking by 0.1 mM DSS. In both B and C, the relative amounts of monomeric gp80 were quantified and plotted below the Western blots. Values are normalized to those of the noncross-linked samples and represent the mean of three experiments.

**FIG. 6. Effects of cold Triton X-100 treatment on gp80 oligomers.** A, cross-linking reactions were performed with 0.1 mM DSS at 4 °C for 30 min with or without prior extraction with 0.2% Triton X-100 for 10 min at 4 °C. The sample extracted with Triton X-100 was separated into a Triton X-100-soluble supernatant (S) and a Triton X-100-insoluble pellet (P). Equal proportions of each sample were separated in a 5% gel, blotted, and probed for gp80. B, cells were first cross-linked with 0.1 mM DSS at room temperature for 30 min, chilled, and then extracted with 0.2% Triton X-100 for 10 min at 4 °C. Equal proportions of the Triton X-100 soluble (S) and insoluble (P) fractions were probed for gp80 oligomers.

decreased the level of cross-linked gp80 species and increased the amount of monomers (Fig. 6A). These results indicate that gp80-gp80 interactions were sensitive to cold Triton X-100.

To determine whether the stabilized oligomers were associated with raft-like structures, the extracted and cross-linked sample was separated into Triton X-100-soluble and -insoluble fractions. The cross-linked dimers and trimers were preferentially associated with the Triton-insoluble fraction, whereas the supernatant contained predominantly gp80 monomers (Fig. 6A). When cells were cross-linked first and then extracted, the levels of dimers, trimers, and species trapped in the stacking gel were −2.3, −3, and 9-fold higher, respectively, in the Triton X-100-insoluble fraction versus the supernatant (Fig. 6B). In contrast, the gp80 monomers were 1.2-fold higher in the supernatant. Therefore, gp80 oligomerization apparently enhanced its partitioning into the raft-like domains.

**Effects of Sterol Sequestration and Cleavage of the Phospholipid Anchor on Gp80 Oligomerization**—To assess the relationship between gp80 oligomerization and raft localization, we performed sterol sequestration treatments that were previously shown to disperse clusters of other GPI-anchored proteins (34). Cell aggregates were treated with low levels of digitonin or filipin to sequester sterols and then cross-linked with DSS. Although sterol sequestration induced cell rounding, neither digitonin nor filipin affected gp80 cross-linking, whereas Triton X-100 partially dissociated the oligomers (Fig. 7, A–C). Immunostaining of these cells revealed discrete patches of gp80 at both cell-cell contacts and noncontact surfaces, further suggesting that gp80-gp80 interactions resisted sterol sequestration.

The above observation suggested that sterol-gp80 interactions might not be a major contributor to the stability of gp80 oligomers. To assess whether direct interactions between extracellular domains could mediate gp80 oligomerization in the absence of membrane association, we analyzed gp80 molecules released from the cell surface through cleavage of their phospholipid anchors by an endogenous phospholipase (35). Gp80 molecules released into the medium were isolated in an S-100
cells were deposited on coverslips, fixed, and stained for gp80. Bars A cal images of treated cells. Following the treatments described in filipin for 20 min prior to cross-linking with 0.1 mM DSS. Proteins were are indicated.

fraction, which represented ~15% of the cellular gp80. After concentration, proteins were cross-linked with either DSP or DSS. Dimer and trimer species of gp80 were detected in both cases (Fig. 7D). Direct interactions between the extracellular domains of gp80 were evidently sufficient for oligomerization.

Gp80 Oligomerization Is Mediated Predominantly by cis-Interactions—The gp80 homophilic binding site that mediates trans-interactions has been mapped (25) and is recognized by the mAb 80L5C4 (20). We investigated the contribution of this site to gp80 oligomerization by incubating 10-h cells with equivalent amounts of either gp80 Fab or control Fab, followed by cross-linking. Anti-gp80 Fab consistently reduced the upper dimer band to the background level (Fig. 8A). However, the other cross-linked species were unchanged, suggesting that the lower dimer (d1) and the other oligomers were maintained by cis-interactions, whereas the upper dimer (d2) was formed by trans-interactions.

To assess gp80 oligomerization on single cells, cells were dissociated with high shear forces in 10 mM EDTA. The cross-linked gp80 species persisted after the dissociation (Fig. 8B). Even with the addition of latrunculin B, the lower dimer (d1) and other oligomers remained, whereas the upper dimer (d2) was substantially reduced.

The presence of such gp80 cis-oligomers should permit gp80 capping through bivalent mAb cross-linking alone. Indeed, exposure of live single cells to anti-gp80 mAb alone readily induced the redistribution of gp80 into caps (Fig. 8C). Gp80 displayed an even membrane distribution on fixed and untreated single cells (Fig. 8D). Together, these results indicate that gp80 oligomers occurred predominantly in cis.

Elevated Gp80 Expression Is Accompanied by Increases in Gp80 Oligomerization—Clustering of GPI-anchored proteins into rafts through lipid interactions with their anchors was found to be independent of protein expression (5, 6). However, if oligomerization is mediated by direct interactions between the extracellular domains of gp80, it may show a dependence on the level of gp80 expression. To distinguish between these possibilities, we cross-linked cells with 0.1 mM DSS from 0 to 12 h of development, when gp80 expression increases from background to maximal levels (36). From 4 to 12 h, total gp80 expression increased ~10-fold. The cross-linked species also increased, whereas the level of gp80 monomers formed a plateau from 8 to 12 h (Fig. 9, A and B). Assuming that the difference between total gp80 and monomeric gp80 corresponded to the amount of gp80 oligomers, the percentage of gp80 oligomers increased linearly from 20 to 55% over the 10-fold range of gp80 expression between 4 and 12 h (Fig. 9C). From 6 to 12 h, the putative trans-dimers increased by ~1.8-fold, but the putative cis-dimers and trimers each increased by ~3-fold (Fig. 9D). Similar results were obtained after cross-linking with 1 mM DSS (data not shown). The data indicate that direct interactions between gp80 molecules are important for gp80 oligomerization associated with cell-cell adhesion.

**DISCUSSION**

In this paper, we have isolated and analyzed low density plasma membrane fragments that display characteristics of membrane rafts. Additionally, the phospholipid-anchored cell adhesion molecule gp80 was found to form stable oligomers that partitioned preferentially into raft-like membranes. Our results reveal the coordination of multiple molecular interactions as gp80 assembles into adhesion complexes during Dictyostelium development. A model is proposed for this process (Fig. 10).

The existence of rafts in Dictyostelium plasma membrane is evident from the isolation of similar raft-like complexes by distinct methods. In this study, low density plasma membrane fragments, isolated with a detergent-free method, have been shown to share many properties with Dictyostelium TIFF (14). Both complexes contain high lipid contents and are especially enriched in sterols. Although they exclude most membrane protein species, they both contain gp80, gp138 family members, DdCD36, actin, and ponticulin. Gp138 and ponticulin were identified in TIFF after performing the molecular weight corrections described in the legend to Fig. 1. The main proteins in
the membrane fragments are phospholipid-anchored, as such anchorage has been shown for gp80 (23, 24) and ponticulin (37) and predicted for gp138 family members (38). The enrichment of sterols and phospholipid-anchored proteins in these membranes, together with their insolubility in cold Triton X-100, suggest that they have the liquid-ordered structure characteristic of rafts (7, 8).

The raft-like domains likely play a key role in the assembly and function of the cell adhesion molecule gp80. However, raft-like fragments can be isolated from gp80-null cells, indic-
cating that the formation of rafts is independent of gp80. During development, gp80 molecules are recruited into rafts through interactions between their phospholipid anchor and the sterol-rich domains. 3) As the overall level of gp80 increases, the elevated concentration of gp80 molecules in rafts leads to local cis-interactions via their extracellular domains. 4) The affinity of gp80 for rafts is increased upon oligomerization. 5) gp80 cis-oligomers accumulate as development proceeds. 6) The presentation of cis-oligomers in rafts facilitates trans-dimerization required for cell-cell adhesion. The combination of trans- and cis-interactions promotes the cross-linking and coalescence of the adhering rafts. 7) Cytoskeleton association with the enlarged rafts contributes to the further expansion of the gp80 adhesion complexes.

Our chemical cross-linking studies detected relatively stable gp80 oligomers on aggregating cells during development. Cis-interactions between gp80 molecules are probably the major force stabilizing these oligomers as depicted in step 3 of our model. Gp80 is known to mediate adhesion through trans-dimerization at a single homophilic binding site that we have mapped (25). Exposure to antibodies that block this site diminishes one of the two detected gp80 dimers, thus identifying it as the gp80 trans-dimer. The resistance of the other oligomers to both this treatment and cell dissociation indicates that they can be maintained by cis-interactions alone. These cis-oligomers also display independence from the trans-dimer during development, as they accumulate to a much greater extent. Moreover, immunoelectron microscopy detected clusters of gp80 on the surface of single cells (39). Most clusters have diameters from 30 to 70 nm, values remarkably close to sizes ascribed to vertebrate rafts (6, 40). Consistent with this observation, gp80 redistribution into caps can be induced by exposure of live single cells to mAb alone. Since capping of membrane proteins normally requires multivalent cross-linking (41), capping in response to a bivalent cross-linker implicates the prior existence of gp80 clusters.

The gp80 oligomers are partitioned preferentially within rafts. Relative to monomers, the oligomers are enriched in rafts isolated either with or without detergent. Moreover, −55% of gp80 molecules form oligomers at a stage when 50–55% of gp80 is resistant to Triton X-100 (14). Since gp80 oligomerization was not concomitant with its recruitment into rafts, the pref-
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