Involvement of a Triton-Insoluble Floating Fraction in *Dictyostelium* Cell-Cell Adhesion*

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Running title: Structure and function of *Dictyostelium* TIFF.

* This work was supported by Operating Grant MT-6140 from the Canadian Institutes of Health Research.

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Abbreviations used: TIFF, Triton-insoluble floating fraction; CHIFF, CHAPS-insoluble floating fraction; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight; GFP, green fluorescent protein; RMLC, regulatory myosin light chain; GPI, glycosylphosphatidylinositol, EM, electron microscopy.
SUMMARY

We have isolated and characterized a Triton-insoluble floating fraction (TIFF) from *Dictyostelium*. Ten major proteins were consistently detected in TIFF and six species were identified by mass spectrometry as actin, porin, comitin, regulatory myosin light chain, a novel member of the CD36 family, and the phospholipid-anchored cell adhesion molecule gp80. TIFF was enriched with many acylated proteins. Also, the sterol:phospholipid ratio of TIFF was 10-fold higher than that of the bulk plasma membrane. Immuno-electron microscopy showed that TIFF has vesicular morphology and confirmed the association of gp80 and comitin with TIFF membranes. Several TIFF properties were similar to those of *Dictyostelium* contact regions, which were isolated as a cytoskeleton-associated membrane fraction. Mass spectrometry demonstrated that TIFF and contact regions shared the same major proteins. During development, gp80 colocalized with F-actin, porin and comitin at cell-cell contacts. These proteins were also recruited to gp80 caps induced by antibody crosslinking. Filipin staining revealed high sterol levels in both gp80-enriched cell-cell contacts and gp80 caps. Moreover, sterol sequestration by filipin and digitonin inhibited gp80-mediated cell-cell adhesion. This study reveals that *Dictyostelium* TIFF has structural properties previously attributed to vertebrate TIFF and establishes a role for *Dictyostelium* TIFF in cell-cell adhesion during development.
INTRODUCTION

Cell membranes are thought to exist primarily in a fluid, liquid crystalline phase. However, certain membranes display elevated acyl chain order and exist in a liquid-ordered phase. These membranes exhibit Triton-X 100 insolubility and can be separated from other insoluble cellular material by floatation into density gradients after isopycnic centrifugation (1-3). We will refer to these membranes as a Triton X-100 insoluble floating fraction (TIFF). TIFF has distinctive structural properties and is involved in a variety of cellular functions. TIFF is typically isolated as membrane vesicles (4-6). These membranes are enriched in lipids, such as cholesterol and those with saturated acyl chains, which are expected to pack closely within the liquid-ordered environment (7). In addition, many cell membrane-associated structural and signaling proteins have been found in TIFF (8). TIFF proteins are often anchored to the membranes through a lipid moiety (7).

TIFF was originally characterized in vertebrate cells (4, 5). However, TIFF analyses have been extended to yeast and Drosophila (6, 9, 10), and a CHAPS-insoluble floating fraction (CHIFF) has been reported in Dictyostelium discoideum (11). The physiological importance of TIFF has been shown by perturbation of TIFF protein activities by cholesterol sequestration and through the co-localization of TIFF components at sites of cellular activity. Processes that involve TIFF include cellular trafficking (7), T-cell signaling (12-16), integrin signaling (17, 18), and bacterial interactions with macrophages (19) and mast cells (20).

Dictyostelium is a favorable model organism for the study of plasma membrane structure and function. Dictyostelium is amenable to both biochemical and molecular genetic analyses of its cellular and developmental processes. During Dictyostelium development, unicellular amoeboid cells aggregate through chemotaxis towards cAMP and embark on a multicellular developmental program (21). Three components of the cAMP signaling pathway (the cAMP receptor cAR1, adenylate cyclase and the cell surface phosphodiesterase) are found in CHIFF, suggesting that they are components of specialized microdomains on the plasma membrane (11).
Multicellularity in *Dictyostelium* is maintained by several cell adhesion molecules, including DdCAD-1/gp24 (22-24), gp150/LagC (25-27), and gp80 (28, 29). gp80 is expressed during the aggregation stage of development (30-32) and mediates the so-called contact sites A by a Ca$^{2+}$/Mg$^{2+}$-independent homophilic binding mechanism (33-35). gp80 is both necessary for strong adhesion during development (36-38) and sufficient for the aggregation of otherwise non-adhesive vegetative cells (39, 40).

It is apparent that gp80-gp80 interactions mediate cell-cell adhesion among *Dictyostelium* cells. However, structural details of gp80 adhesion complexes are largely unknown. Intriguingly, gp80 is enriched in Triton X-100-insoluble, cytoskeleton-associated contact regions (41). This subcellular fraction contains stacked membranes with dimensions of intact cell-cell contacts and can only be isolated after the cell aggregation stage of development. Considering that gp80 is phospholipid-anchored (42, 43), we hypothesized that these Triton X-100-insoluble contact regions may be a form of TIFF and that gp80 adhesion complexes may be organized as distinct membrane domains within the plasma membrane.

In the present study, we have isolated *Dictyostelium* TIFF, characterized its morphology, identified its protein constituents and analyzed its lipid composition. We demonstrate that TIFF membranes share many common physical and biochemical properties with the Triton X-100-insoluble contact regions. Furthermore, a role for TIFF components during gp80-mediated adhesion is established through colocalization, co-capping and adhesion perturbation studies.
EXPERIMENTAL PROCEDURES

Cell Growth and Development

Dictyostelium cells, including the wild-type axenic strain AX2 and the gp80-null strain GT10 (36), were cultured either in association with Klebsiella aerogenes or axenically in HL5 liquid media (44). For development, cells at the late exponential growth phase were collected, washed and resuspended at 1.5 x 10^7 cells/ml in MCM buffer (45) and then shaken at 180 rpm. To stimulate gp80 expression, cells were pulsed with cAMP at a final concentration of 2 x 10^{-8} M every 7 min. Cells were also developed in MCM buffer on coverslips.

Construction of GFP-comitin Vector and Cell Transformation

A GFP-comitin construct was produced by end-filling an EcoRI fragment containing the comitin cDNA (46), followed by subcloning into the end-filled HindIII site of the Dictyostelium expression vector pBS18-74E (obtained from Dr. R. Firtel, University of California at San Diego, La Jolla, USA). Expression of comitin was under the control of the actin15 promoter. The BamHI/BglII fragment of pBS-GFPII (obtained from Dr. H. MacWilliams, Ludwig Maximillian University, Munich, Germany), containing the entire coding region of GFP, was then subcloned into the BglII site of comitin in the resulting plasmid. Hence, GFP was inserted in-frame between Arg15 and Ser16 of comitin. Plasmid DNA was introduced into AX2 cells by the calcium phosphate co-precipitation method (47). Stable transformants were selected and maintained in HL5 medium containing 20 µg/ml of G418.

Isolation of TIFF

TIFF was isolated from cell aggregates that were collected at 12 h of development and gently resuspended at 5 x 10^7 cells/ml in cold buffer 1 (40 mM sodium pyrophosphate, 0.4 mM DTT, 0.1 mg/ml PMSF, 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 centrifuged at 14,500g and the
pellet was washed with buffer 1, centrifuged at 14,500g, washed with buffer 2 (1 mM EGTA, 5
mM Tris-HCl, pH 7.6) and centrifuged at 14,500g. The resulting pellet was resuspended in
buffer 2 and mixed at a 1:2 ratio with 60% (w/w) sucrose, placed at the bottom of a centrifuge
tube, and then overlaid with a 11-ml continuous gradient of 10-40% (w/w) sucrose. The
gradients were centrifuged at 120,000g for 15-17 h at 2°C using a Beckman SW40 rotor. The
TIFF material banded at ~34% sucrose was collected. In subsequent experiments, TIFF was
isolated in discontinuous gradients from the interface between 28 and 38% sucrose. TIFF
collected from the sucrose gradients was washed with 20 mM sodium phosphate buffer, pH 7.6,
and then pelleted by centrifugation at 39,000g for 20 min at 2°C.

Isolation of Plasma Membranes and Triton X-100-Insoluble Contact Regions

Plasma membranes were isolated using the aqueous two-phase polymer system (48).
Triton X-100-insoluble contact regions were isolated according to Ingalls et al. (41). Cells were
treated with 0.2% Triton X-100 at 4°C and then centrifuged at 4000g. The pellet was washed
and resuspended in buffer 2 and then layered on the top of a discontinuous gradient of 5.5 ml
50% and 5.5 ml 60% (w/w) sucrose in 20 mM phosphate buffer, pH 6.8, for centrifugation at
120,000g for 3 h at 2°C. The material banded at the interface was collected, washed, and
dialyzed in a cytoskeleton depolymerizing solution containing 0.1 mM EDTA, 0.2 mM sodium
phosphate buffer, pH 7.6. The dialyzed material was pelleted, resuspended in 1.5 ml of buffer 2
and layered on top of an 11-ml continuous gradient of 26-51% (w/w) sucrose. The gradient was
centrifuged at 120,000g for 3 h at 2°C. Membranes banded at 32-34% sucrose were collected.

Gel Electrophoresis

Protein concentrations were determined using the bicinchoninic acid protein assay kit
(Pierce). For SDS-PAGE, proteins were solubilized and reduced by boiling for 5 min in 3%
(w/v) SDS, 3 M urea and 5% (v/v) β-mercaptoethanol and then separated in a slab gel (49). For
immunoblot analysis, bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence kit (Amersham). Immunoblots were quantified using the Bio-Rad Fluor-S Max multiimager system.

**MALDI-TOF Mass Spectrometry**

For protein identification by mass spectrometry, silver-stained gel bands were excised, macerated, reduced, alkylated, and then digested with trypsin. The tryptic peptides were extracted using the protocol of Shevchenko et al. (50). The peptides were purified using Sephasil C18 resin, applied to MALDI plates, and mixed with a matrix solution of α-cyano-4-hydroxy-trans-cinnamic acid (Sigma) at 20 mg/ml in 50% (v/v) acetone and 50% (v/v) isopropanol. The samples were dried and then subjected to mass spectrometry. Peptide masses were determined using a PerSeptive Biosystems Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems, Inc.) in the linear mode, with 92% grid voltage, 0.15% guide wire voltage, laser intensities between 1600 and 2100, and delayed extraction of 200 ns. Trypsin autolysis products and matrix molecules were used for calibration. The remaining masses were submitted to the ProFound search engine (http://prowl.rockefeller.edu/cgi-bin/ProFound) and the Protein Prospector search engine (http://prospector.ucsf.edu/ucsfbin/msfit.cgi) for matches. Search parameters were held constant, including tolerance for peptide mass error of ±1 Da, tolerance for protein mass error of ±10 kDa from apparent molecular weights determined by SDS-PAGE, and a maximum of one missed cut per peptide.

**Immuno-gold Electron Microscopy**

Cells expressing GFP-comitin were collected at 10 h of development for TIFF isolation. TIFF membranes were incubated overnight with anti-gp80 mAb 80L5C4 (29) or anti-GFP rabbit antibody (Molecular Probes) in TBS (20 mM Tris, pH 7.6, 137 mM NaCl) plus 0.1% (w/v) BSA, with rotation at 4°C. After washing, samples were incubated with either goat antimouse or goat anti-rabbit antibodies conjugated to 10-nm gold (Sigma) at 1:10 dilution in TBS
plus 0.1% (w/v) BSA. Samples were rotated overnight at 4°C. After four washes, pellets were fixed overnight at 4°C with 1% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. After several washes, the pellets were incubated in 1% (w/v) OsO4 in phosphate buffer for 30 min at room temperature. Samples were dehydrated with an ethanol series followed by propylene oxide and then embedded in Spurr’s standard resin. Ultra-thin sections were cut with a diamond knife and then stained with uranyl acetate and lead citrate prior to examination under a Hitachi 8600 analytical transmission electron microscope.

**In situ Triton X-100 Extraction**

To perform Triton X-100 extraction of living cells, cell aggregates on coverslips were chilled to 4°C by replacing the MCM buffer with cold buffer 1 and placing the coverslips on an ice water bath for 15 min, Triton X-100 was added to a final concentration of 0.2% (v/v). After 5 min, the Triton X-100 solution was aspirated and the coverslips were fixed with cold 10% (v/v) formaldehyde for 10 min on ice, followed by 3.7% (v/v) formaldehyde at 22°C for 10 min. Cells were stained and prepared for confocal microscopy.

**Metabolic Labeling with Palmitic Acid**

Cells were developed at 2 x 10^7 cells/ml for 2 h in 17 mM phosphate buffer, pH 6.1, containing streptomycin (0.5 mg/ml). Cells were collected and resuspended in phosphate buffer containing 0.1 mCi/ml of [9,10-^3^H]palmitic acid (New England Nuclear) and developed for another 6 h with cAMP pulsing. Cell aggregates were collected and proteins in different subcellular fractions were analyzed by SDS-PAGE. Protein blots were coated with EN^3^HANCE spray (New England Nuclear) and exposed to Biomax MR film (Kodak) at -70°C for 3 weeks.

**Lipid Analyses**

Samples were analyzed by gas-liquid chromatography as described previously (51). Briefly, sample lipids were extracted with chloroform/methanol (2:1 v/v) and digested with
phospholipase C (Clostridium welchii). The mixture was extracted with chloroform/methanol (2:1 v/v) containing 100 µg of tridecanoylglycerol as an internal standard. The samples were then incubated in SYLON BFT plus 1 part dry pyridine for 30 min at 20°C. All extracted lipids are converted into neutral species by this procedure and they were quantified after separation on a non-polar capillary column.

**Cell Staining**

Cells were developed for 12 h on coverslips and then fixed, stained and mounted as previously described (52, 53). Samples were first incubated with primary antibodies against gp80 or porin, followed by Alexa 568/488-conjugated secondary antibodies (Molecular Probes) at 1:300 dilution. Filamentous actin (F-actin) was stained with fluorescein-phalloidin (Molecular Probes) at 1:10 dilution. Laser scanning confocal microscopy was performed using a Zeiss Axiovert 135 inverted microscope equipped with a 63X Neofluor objective and a LSM 410 confocal attachment. Detection was maintained within the range of the gray scale to prevent signal saturation.

For filipin staining, cells were grown axenically, developed on coverslips and then fixed in 3.7% (v/v) formaldehyde in MCM buffer for 15 min at room temperature. Cells were incubated with 0.025% (w/v) filipin in MCM buffer for 15 min at room temperature, washed, and then mounted for epifluorescence microscopy.

**Capping of Cell Surface Proteins**

Cells were developed in suspension for 6-8 h with cAMP pulsing. Cell aggregates were dispersed and deposited on coverslips in MCM buffer (3 x 10^5 cells/cover slip). After 10 min, anti-gp80 mAb or polyclonal antibodies were added to cells for 15 min at room temperature. The coverslips were washed gently with two changes of 10 ml MCM buffer for 3 min. Alexa 568-conjugated secondary antibodies were added at 1:50 dilution for 15 min, followed by two washes. The cells were fixed at 40 min after the initial addition of the primary antibody. To
identify co-capped molecules, fixed cells were stained with appropriate antibodies.

**Cell Cohesion and Cell Dissociation Assays**

Cell cohesion was assayed as described previously (54). Alternatively, the effects of various reagents on cell cohesion were assayed by measuring aggregate dissociation under high shear force. Cells were cultured on bacteria plates and then collected for development in liquid culture at $2 \times 10^7$ cells/ml. At different time points, cell aliquots were taken and diluted in 17 mM phosphate buffer, pH 6.1, plus the reagent under test. Stock solutions of filipin and digitonin were prepared fresh in DMSO prior to each experiment. Equivalent amounts of DMSO were added to control samples. Cell aggregates were incubated in various reagents for 10 min at room temperature with gentle shaking. Shear force was then applied by continuous vortexing for 30 sec using a Vortex Genie 2 at setting 8. Cell dissociation was quantified by counting cells with a hemacytometer. Singlets, doublets and triplets were scored as dissociated cells and the percent cell dissociation was calculated relative to the number of cells obtained at 0 h which was $\sim 2 \times 10^6$ cells/ml.
RESULTS

Isolation and Characterization of TIFF

TIFF was isolated from Dictyostelium cells after 10 to 12 h of development in liquid medium. After fractionation of detergent-insoluble material in a continuous sucrose density gradient (Fig. 1A), TIFF formed a sharp band at ~34% sucrose (Fig. 1A, lane 9), whereas the cytoskeleton pelleted (Fig 1A, lane 13). The highly resolved banding pattern suggested that TIFF had a relatively homogeneous composition. This isolation protocol routinely yielded 0.8 to 0.9 mg of TIFF protein from 10^10 cells.

Silver staining of TIFF proteins revealed a highly reproducible profile (Fig. 1B). The ten most strongly stained bands were designated ‘t’ followed by their apparent molecular mass in kDa. Protein identification was attempted using MALDI-TOF mass spectrometry and database search engines. Protein bands designated t103, t88, t43, t28, t23 and t20 were identified to be the Dictyostelium expressed sequence tag (EST) C84888, gp80, actin, porin, comitin and regulatory myosin light chain (RMLC), respectively (Fig. 1C). These proteins produced the strongest mass spectra of the ten analyzed. As an example, the mass spectrum of t103 is shown (Fig. 1D). Their identifications displayed similar ranks and scores, based on two search engines. They each had similar sequence coverage with their matches and displayed the expected electrophoretic mobility.

Since the identification of t103 was limited to one search engine, a post source decay (PSD) analysis was performed (Fig. 1D, E). Every PSD product of the 2498 Da peptide corresponded to the predicted sequence of the EST, thus confirming the match (Fig. 1E). To annotate the EST, the position specific iterated basic local alignment search tool was used to search for similar proteins. The search results showed that the C-termini of members of the CD36 family had the highest scores and t103 was, therefore, designated DdCD36. The identity of gp80, actin, porin and comitin were confirmed by Western blot analysis (Fig. 1F).

Since proteins anchored to the plasma membrane via a lipid moiety are known to be
associated with TIFF (7), we labeled cells metabolically with $[^3]$H]palmitic acid and determined whether labeled proteins were enriched in the TIFF fraction. Labeled cells were fractionated into three parts: the Triton X-100 soluble fraction, the Triton X-100 insoluble pelleted fraction after floatation centrifugation, and TIFF. Equal amounts of protein from each fraction were separated by SDS-PAGE and compared after fluorography (Fig. 2). TIFF was enriched with many palmitoylated proteins. Several intensely labeled species displayed gel mobilities corresponding to gp80, t46, actin, RMLC and t18, suggesting that these proteins or some co-migrating species were heavily palmitoylated.

**Enrichment of Sterols in TIFF**

Since membrane insolubility in cold Triton X-100 is a characteristic of liquid ordered membrane structure (1-3), we expected Dictyostelium TIFF to be enriched in lipids conforming to this structure. Total lipids in plasma membranes and TIFF were analyzed and compared (Table 1). TIFF contained a 15-fold higher sterol level and a 1.5-fold higher phospholipid level than the plasma membrane. The elevated lipid-to-protein ratios in TIFF probably accounted for its low density (1.13-1.14 g/ml). The sterol-to-phospholipid ratio in TIFF was ~10-fold higher than that of plasma membranes. The sterol species in TIFF were identified to be stigmasterol (78%), campesterol (14%) and sitosterol (8%) based on column retention times. The stigmasterol species was likely $\Delta^{22}$-stigmasten-3β-ol because it accounts for 88% of the sterols in Dictyostelium cells (55). Both TIFF and plasma membranes had similar sterol compositions.

**Morphological Characterization of Dictyostelium TIFF**

To assess the effects of Triton X-100 extraction on the plasma membrane of cells, the subcellular distribution of gp80 was examined, since it was the most prominent TIFF protein and a good plasma membrane marker. Cells developed on coverslips were extracted with Triton X-100 and then fixed prior to staining with anti-gp80 mAb. Confocal microscopy revealed a lattice-like pattern of gp80 staining along the cell periphery, suggesting the formation of gp80-
enriched vesicular structures on the plasma membrane (Fig. 3A).

The morphology of TIFF was examined by electron microscopy. Membrane vesicles with diameters ranging from 0.1 to 1.0 µm were observed (Fig. 3B to D). These membranes often stacked upon one another to form multi-layered structures similar to those observed for the Triton X-100-insoluble contact regions (41). The samples appeared relatively homogeneous and were devoid of other membranous organelles.

Since antibodies against comitin showed cross-reactivity with several protein bands, transformants expressing GFP-comitin were made. GFP-comitin could be detected specifically using an anti-GFP antibody and immunoblot analysis showed that the fusion protein was enriched in TIFF (data not shown). Immuno-gold labeling of gp80 and GFP-comitin showed the association of both proteins with the TIFF membranes (Fig. 3B, C). gp80 displayed a patchy distribution, which was observed over a 10-fold range of antibody concentrations, while GFP-comitin was distributed more evenly along the membranes.

**Co-capping of F-actin, Comitin and Porin with gp80**

Since gp80 is the predominant protein in TIFF and Triton-insoluble gp80 represented 50-55% of total cellular gp80, it was important to determine whether gp80 existed together with other TIFF components as complexes in the plasma membrane of intact cells. If this was the case, clustering of gp80 on living cells by antibody crosslinking should lead to the co-capping of other TIFF proteins. Cells were induced to form gp80 caps, and double immunofluorescence labeling revealed the co-capping of both porin and F-actin with gp80 (Fig. 4A, B, E, F). GFP-comitin was also found to co-localize with gp80 caps (Fig. 4C, D). Therefore, porin, F-actin and comitin were membrane-associated components that could be induced to form large complexes with gp80 via antibody crosslinking. In addition, crosslinking of gp80 induced cell rounding, indicative of cytoskeleton reorganization.

**Subcellular Localization of Sterols by Filipin Staining**
Results presented earlier revealed high levels of sterols in the gp80-enriched TIFF membranes (see Fig. 1 and Table 1). The relationship between gp80 and sterols was examined further by capping gp80 on living cells, which were then fixed and stained with filipin. Filipin staining revealed a high concentration of sterols associated with gp80 caps (Fig. 5A, B). In addition to gp80 caps, co-localization of sterols with gp80 at cell-cell contacts was observed (Fig 5C, D). To avoid perturbations by antibody exposure, cells were also stained with filipin without prior incubation with anti-gp80 mAb. Epifluorescence microscopy again revealed high sterol levels at cell-cell contacts between aggregating cells (Fig. 5E). These results indicate that sterols are enriched in cell-cell contact regions as well as TIFF.

**Co-localization of TIFF Proteins at Cell-Cell Contacts**

The subcellular distribution of TIFF proteins in aggregating cells was examined by confocal microscopy. Co-localization of gp80 and F-actin was evident at cell-cell contact regions (Fig. 6A, B). Double staining for gp80 and porin also revealed their co-localization at cell-cell contact regions (Fig. 6C, D). In addition, porin displayed punctate cytoplasmic staining, consistent with its mitochondria association (56). Finally, GFP-comitin was also co-localized with gp80 at cell-cell contact regions (Fig. 6E, F). In addition to contact regions, GFP-comitin displayed punctate cytoplasmic staining.

**Comparison of Triton-Insoluble Contact Regions with TIFF**

The Triton X-100-insoluble contact regions were also enriched in gp80 (41), suggesting that this subcellular membrane fraction might be related to TIFF. To compare the properties and composition of these two membrane fractions, contact regions were isolated from cell aggregates at 10-12 h of development (Fig. 7). In contrast to TIFF, the contact regions co-fractionated initially with the cytoskeleton and displayed a relatively high density after equilibrium centrifugation. Following actin depolymerization, the cytoskeleton-free contact regions shifted to a lower density and formed a highly resolved band between 32-34% sucrose after equilibrium
centrifugation through a continuous sucrose gradient. This density was similar to that of TIFF. The silver-stained protein profile of the purified contact regions was also similar to that of TIFF (Fig. 7). Protein bands were excised from the gel, digested with trypsin and analyzed by MALDI-TOF mass spectrometry. The ten proteins with the strongest mass spectra were designated ‘cr’ followed by their apparent molecular mass in kDa. The contact region proteins cr22, cr28, cr42, cr88 and cr110 were identified as comitin, porin, actin, gp80 and DdCD36, respectively (Fig. 7). Proteins lacking positive identifications were also shared between the TIFF and contact region fractions. Lipid analysis showed that the isolated contact regions were also highly enriched in sterols (data not shown). Thus, TIFF and Triton X-100-insoluble contact regions share many common properties although the latter is associated with the cytoskeleton.

**Sterol Sequestration Perturbs both TIFF Recovery and Cell-Cell Adhesion**

In vertebrates, cholesterol is required for the structural integrity of TIFF (57). Although *Dictyostelium* cells do not synthesize cholesterol, the above results show that TIFF and intact cell-cell contacts are enriched in sterols. Both filipin and sub-critical micelle concentrations of digitonin are known to sequester sterols within *Dictyostelium* plasma membranes (58). Therefore, we tested whether these reagents had an effect on the structural integrity of TIFF. When cell aggregates were treated with either 0.01% digitonin or 0.004 % filipin prior to Triton X-100 extraction, TIFF recovery was reproducibly reduced to less than 10% of their respective controls (Fig. 8A).

The effects of sterol sequestration on gp80-mediated cell-cell adhesion were also examined. The EDTA-sensitive cell adhesion sites mediated by DdCAD-1 are fully developed during the first few hours of development (23, 59) and they can be easily disrupted by mechanical force. The gp80-dependent EDTA-stable adhesion sites appear later and confer stronger adhesion among cells (36-38). At first, cell-cell adhesion was monitored using the established cell reassociation assay (54). At low concentrations when cell viability was not affected, neither digitonin nor filipin had a significant effect on cell reassociation (data not...
shown). Since similar concentrations have perturbed cellular distributions and functions of vertebrate TIFF proteins (60, 61), it was possible that a more stringent assay was needed to detect perturbation of the strong adhesion mediated by gp80. Therefore, a cell dissociation assay was developed to monitor the resistance of cell aggregates to high shear forces. At 0 and 3 h of development, cells were subjected to high shear forces and microscopic examination revealed complete cell dissociation at both time points (Fig 8B). The small drop in the cell dissociation curve at 3 h was probably due to cell loss via attachment to the container surface with development. After 6 h, aggregates that resisted shear forces were observed and the number of dissociated cells diminished. Cell-cell adhesiveness was further strengthened by 9 h. The resistance of cell dissociation coincided temporally with the accumulation of high levels of gp80, suggesting that this strong cell-cell adhesion was mediated by gp80. Cell aggregates of gp80-null cells, formed through the EDTA-sensitive adhesion molecules, were subjected to high shear forces and microscopic examination revealed complete cell dissociation at all developmental time points (Fig 8B).

Next, we determined whether the gp80-dependent resistance to shear forces was sensitive to treatment with low concentrations of filipin and digitonin. Incubation of cells with either 0.005% filipin or 0.001% digitonin for 10 min led to high levels of cell dissociation at all time points (Fig 8C). Sterol sequestration is a common activity of these agents that otherwise have dissimilar cellular effects (62). To control for other effects, such as the formation of transmembrane pores, cells were incubated with 0.001% Triton X-100. This treatment had no effect on the resistance of cell aggregates to shear forces. Both filipin and digitonin treatments yielded results similar to those obtained with the gp80-null cells, consistent with the idea that the adhesive function of gp80 is sensitive to sterol sequestration.
DISCUSSION

We have isolated and characterized TIFF from *Dictyostelium* cells. In many ways, *Dictyostelium* TIFF is similar to other detergent insoluble floating fractions. Its protein components have counterparts in vertebrate TIFF’s, such as CD36, actin, porin and RMLC (8). *Dictyostelium* TIFF is highly enriched in gp80 (an analogue of GPI-anchored proteins), palmitoylated proteins and sterols, as found in vertebrate TIFF (1, 7). Furthermore, *Dictyostelium* TIFF has the common vesicular TIFF morphology (4-6).

High levels of cholesterol and ergosterol have been found in TIFF (4, 6, 9). Although *Dictyostelium* cells synthesize primarily stigmasterol, campesterol and sitosterol, the sterol content of *Dictyostelium* TIFF is 15-fold higher than that of plasma membranes. Another notable feature of *Dictyostelium* TIFF is its relatively high sterol:phospholipid molar ratio in comparison to vertebrate TIFF (4) and *Drosophila* TIFF (9). The lipids of *Dictyostelium* CHIFF are also predominantly sterols (11). These high sterol levels may be due to the high level (75-90%) of unsaturated fatty acid side-chains in *Dictyostelium* (55). The unsaturated acyl chains would not conform to the liquid-ordered membrane structure expected for TIFF and CHIFF, but they probably allow membrane fluidity at the normal *Dictyostelium* growth temperature of 22°C. These distinct lipid compositions of the bulk plasma membrane versus the detergent-insoluble floating fractions may have pronounced effects on the structure and function of the sterol-rich domains in *Dictyostelium* membranes.

Although TIFF and CHIFF have a similar lipid composition, these fractions contain different proteins. The cAMP receptor cAR1 is the predominant protein in CHIFF, but it was absent from TIFF, and the opposite is true for the cell adhesion molecule gp80 (11). gp80 and cAR1 also display distinct subcellular distributions during cell aggregation. gp80 becomes concentrated in cell-cell contact regions (52), where it co-localizes with other TIFF proteins and sterols, whereas cAR1 is evenly distributed over the plasma membrane (63). Therefore, the *Dictyostelium* plasma membrane may be organized as a mosaic of domains with specialized
functions. Similarly, different types of detergent-insoluble domains have been shown to segregate within the plasma membranes of vertebrate cells (64, 65). This compartmentalization of specialized membrane components could enable cells to distinguish and efficiently respond to various external stimuli.

Several lines of evidence suggest that TIFF components form membrane domains that are involved in gp80-mediated adhesion. First, gp80 co-localizes with TIFF proteins and sterols in large membrane domains involved in cell-cell contact formation. Second, co-capping experiments show that TIFF components are directly or indirectly linked to gp80. Third, TIFF shares many properties with the Triton-insoluble contact regions (41). Finally, sterol sequestration reduces TIFF recovery and perturbs gp80-dependent intercellular cohesiveness.

How do TIFF properties influence the strong cell-cell binding activity mediated by gp80? Typically, adhesion is strengthened when adhesion molecules cluster to form large complexes that are associated with the cytoskeleton. For transmembrane cell adhesion molecules, their cytoplasmic domains can interact with adapter proteins that link to the cytoskeleton (66-68). In contrast, GPI-anchored adhesion molecules lack direct access to the cytoskeleton. However, TIFF membranes could produce a lipid scaffold for the assembly of both extracellular and cytoplasmic proteins. Specifically, a membrane domain consisting of liquid-ordered lipids may stabilize adhesion complexes by both clustering GPI-anchored adhesion molecules and facilitating their interactions with the cytoskeleton. Indeed, we have observed that gp80-mediated contacts encompass membrane domains, which are enriched in sterols and associated with the cytoskeleton. Moreover, we found that the Triton-insoluble contact regions were basically cytoskeleton-associated forms of TIFF. Therefore, we speculate that sterol sequestration perturbed gp80-mediated adhesion by disrupting liquid-ordered membrane scaffolds within gp80 adhesion complexes.

We have mapped TIFF components to large membrane domains involved in cell-cell contact formation. However, TIFF can also be derived from plasma membrane microdomains termed rafts (7, 62). Thus, it is possible that gp80 adhesion complexes may form from
precursory rafts. In fact, immune-EM studies have revealed gp80 clusters on single cells (69). Most clusters had diameters between 30 and 70 nm, values remarkably close to sizes ascribed to vertebrate rafts (70, 71). Thus, it is likely that gp80 exists within raft-like precursors on the cell surface prior to cell-cell contact. Such rafts may be primed for efficient adhesion complex construction. Preassembly of components within rafts would facilitate rapid complex formation, and avid trans interactions between gp80 clusters could expand the microdomains, eventually leading to the formation of a large cell adhesion complex.

A variety of molecular mechanisms likely underlie cell-cell adhesion. Cell-cell adhesion mediated by lipid-anchored adhesion molecules, such as gp80, is inherently distinct due to the lipid moiety. Many vertebrate cell adhesion molecules are GPI-anchored, including NCAM-120, contactin/F3/F11, axonin-1/TAX-1/TAG-1 (72), T-cadherin (73), neurin-1 (74), LFA-3 (75), and members of the IgLON family (76, 77). In fact, several GPI-anchored cell adhesion molecules have been found in TIFF (78-80). We have demonstrated a role for TIFF in gp80-mediated adhesion that may involve lipid scaffolds and precursory rafts. It would be of interest to determine whether TIFF is involved in adhesion mediated by other lipid-anchored adhesion molecules. Moreover, adhesion complexes constructed from TIFF may display unique regulation and signaling. To explore such signaling and regulatory events, TIFF component interactions that connect gp80 to the cytoskeleton are currently under investigation.

**Acknowledgments.** We thank Drs. M. Opas and R. Reithmeier for advice and discussion, Dr. G. Gerisch for the gift of anti-porin mAb, and Ms. Tak Yee Lam for expert assistance. We also thank Steven Doyle and Battista Calveiri of the EM Laboratory of the Faculty of Medicine, University of Toronto, for assistance with EM and confocal microscopy.
REFERENCES

FIGURE LEGENDS

FIG. 1. TIFF isolation and characterization of TIFF proteins by mass spectrometry. (A) A schematic drawing of the isolation protocol is shown at the top. TIFF was isolated from total Triton X-100 insoluble material by floatation into a continuous sucrose density gradient. Equal volumes of the gradient fractions were analyzed by SDS-PAGE and subjected to silver staining. TIFF floated to ~34% (w/w) sucrose (lane 9, arrowhead), whereas the material associated with the cytoskeleton was pelleted (lane 13). (B) Silver staining profile of TIFF proteins separated on a 12% polyacrylamide gel. The ten most prominent bands are listed at the right. (C) Summary of protein identification data based on mass spectrometry and database searches. All matches were the top ranking *Dictyostelium* proteins from each search engine. The ProFound scores represent probabilities with a maximum score of 1.0. The Protein Prospector utilizes MOWSE scoring that does not have a defined upper limit. Sequence coverage refers to the maximum percent of an identified protein sequence that can be matched to peaks in the mass spectra. The known mass refers to each candidate identified. (D) MALDI-TOF mass spectrum of t103. Peaks that matched (error < 1Da) the masses of peptides encoded by the EST C84888 sequence are marked with an asterisk. Peaks marked with a “T” are trypsin autolysis products. (E) Post-source decay analysis of the 2498 Da peak marked with two asterisks in (D). Decay products are marked with the fragment ions expected from the decay of the corresponding peptide encoded by EST C84888 (error < 1Da). (F) Confirmation of protein identity by immunostaining. Immunoblots of TIFF proteins were stained with antibodies against gp80, actin, porin and comitin.

FIG. 2. Fluorograms of subcellular fractions of cells labeled with palmitic acid. [3H]Palmitic acid was added to cells at 2 h of development and cell aggregates were collected at 8 h, treated with 0.2% Triton X-100 at 4°C and the separated into three fractions: the Triton X-100 soluble fraction (TSF), the Triton X-100 insoluble pelleted fraction (TIPF) collected from the
bottom of the floatation gradient, and TIFF. Equal amounts of protein from these fractions were separated by SDS-PAGE, blotted and prepared for fluorography. The apparent molecular masses in kDa of the major labeled proteins are indicated at the right.

**FIG. 3. Immunolocalization of TIFF components and TIFF morphology.** (A) A confocal image showing the subcellular distribution of gp80 after *in situ* Triton X-100 extraction of 10-h cells. Living cells were treated with the detergent, fixed and then stained with anti-gp80 mAb. Most cells showed punctate staining along the cell periphery. Bar = 5 µm. The inset shows the boxed area at higher magnification, where gp80 displayed a lattice-like staining pattern. Bar = 0.5 µm. (B-D) Electron micrographs of isolated TIFF material. TIFF samples were labeled with primary antibodies followed by 10 nm colloidal gold-conjugated secondary antibodies. (B) Immuno-gold labeling of gp80 on TIFF membranes. (C) TIFF was isolated from cells expressing GFP-comitin and comitin was labeled with antibodies against the GFP tag. (D) To control for antibody trapping, TIFF was probed with secondary antibodies alone. Bars in B-D = 0.25 µm.

**FIG. 4. Co-capping of TIFF components with gp80.** Cells were collected at 10 h of development and incubated with mAb against gp80, followed by a goat anti-mouse secondary antibody. Subsequently, cells were fixed and permeabilized, and then stained with antibodies against porin and actin (GFP-comitin was used to visualize comitin). Double labeled coverslips were examined by confocal microscopy. The membrane domains encompassed by the gp80 caps (A, C, E) were enriched with F-actin (B), GFP-comitin (D), and porin (F), respectively. Bars = 5 µm.

**FIG. 5. Membrane distribution of sterols visualized by filipin staining.** (A, B) Cells were developed on coverslips for 10 h and then incubated with anti-gp80 mAb to induce cap formation. After 40 min of incubation at room temperature, cells were fixed with formaldehyde
and sterols were stained with 0.025% filipin. Confocal images revealed sterol enrichment (A) in membrane domains associated with gp80 caps (B). In addition to gp80 caps, intense filipin staining (C) coincided with gp80 staining (D) in cell-cell contact regions (arrowheads). (E) Fixed cells that were incubated with filipin alone also revealed high levels of sterol at cell-cell contacts (arrowheads). Bars = 5 µm.

**FIG. 6. Co-localization of TIFF proteins with gp80 in cell-cell contact regions.**
Aggregating cells were double stained for several combinations of TIFF protein components. In each vertical pair of panels a region of an aggregation stream, involving contacts between several cells, is shown. Cells were double stained for gp80 (A) and F-actin (B) or for gp80 (C) and porin (D). In addition, transformants expressing GFP-comitin (F) were stained for gp80 (E). Bars = 5 µm.

**FIG. 7. Characterization of Triton X-100-insoluble contact regions.** A schematic drawing of the subcellular fractionation steps is shown at the top. Silver-stained gel profiles of proteins at consecutive steps of the isolation protocol are shown below. Equal protein amounts were loaded per lane, separated by SDS-PAGE, and then subjected to silver staining. Lane 1, whole cell aggregates; lane 2, Triton-insoluble pellet; lane 3, material banded at the 45%/60% sucrose interface; lane 4, post-dialysis pellet; lane 5, Triton-insoluble contact membranes banded at 32-34% sucrose. To the right of the gel is a list of the ten contact region proteins (designated "cr" followed by the apparent molecular mass in kDa) with the strongest mass spectra. In the next column, protein identification based on database searches is shown. In the far right column, matching masses (error < 1.5 Da) between the contact region protein and the corresponding TIFF protein (shown in parentheses). Proteins were compared by first selecting the ten strongest peaks in the contact region protein and scoring matching peaks in the corresponding TIFF protein. Then the opposite operation was performed. Thus, a total of 20 peaks were compared between each pair of proteins and the comparisons are presented as
fractions with the maximum of 20 possible matches as the denominator.

FIG. 8. Effects of sterol sequestration on TIFF and gp80-mediated cell-cell adhesion.

(A) The effect of sterol sequestration on TIFF recovery was examined by incubating cell aggregates with either 0.01% (w/v) digitonin or 0.004% (w/v) filipin for 30 min on ice prior to TIFF isolation. The amounts of protein recovered in TIFF were normalized to their respective control and the data represent the mean ± SD (n = 3). (B) Intercellular cohesiveness assessed by cell dissociation under high shear forces (see Experimental Procedures). Wild-type AX2 cells (o) and gp80-null GT10 cells (l) were developed in liquid culture and collected at different time points for the assay. The data represent the mean ± SD (n = 4). The inset shows the time course of gp80 protein expression in wild-type cells (arrowhead). (C) The effect of sterol sequestration on cell-cell adhesion was evaluated during development. Wild-type cells (AX2) were collected from different developmental time points and incubated for 10 min with 0.001% (w/v) digitonin (l), 0.0005% (w/v) filipin (u), or 0.001% (v/v) Triton X-100 (o). Cells were then subjected to the cell dissociation assay. The data represent the mean ± SD (n = 4). Controls for the DMSO carrier were indistinguishable from the wild-type cells shown in (B).
Table 1. Lipid Compositions of *Dictyostelium* Plasma Membranes and TIFF

<table>
<thead>
<tr>
<th></th>
<th>Sterols (mg/mg protein)</th>
<th>Phospholipids (mg/mg protein)</th>
<th>Sterols/ Phospholipids (mg/mg)</th>
<th>Sterols/ Phospholipids (mol/mol)</th>
</tr>
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<tbody>
<tr>
<td>Plasma</td>
<td>0.236±0.029</td>
<td>0.350±0.055</td>
<td>0.678±0.038</td>
<td>1.26±0.071</td>
</tr>
<tr>
<td>membrane</td>
<td>(1.0)</td>
<td>(1.0)</td>
<td>(1.0)</td>
<td>(1.0)</td>
</tr>
<tr>
<td>TIFF</td>
<td>3.620±0.593</td>
<td>0.541±0.072</td>
<td>6.750±1.15</td>
<td>12.5±2.13</td>
</tr>
<tr>
<td></td>
<td>(15.3)</td>
<td>(1.55)</td>
<td>(9.96)</td>
<td>(9.92)</td>
</tr>
</tbody>
</table>

The values represent the means ± S.D. (for plasma membrane, n=3; for TIFF, n=5). Relative amounts are indicated in parentheses.
Figure 1 (Harris et al.)
Figure 7 (Harris et al.)
Figure 8 (Harris et al.)
Involvement of a triton-insoluble floating fraction in dictyostelium cell-cell adhesion
Tony J.C. Harris, Donald E. Awrey, Brian J. Cox, Amir Ravandi, Adrian Tsang and Chi-Hung Siu

J. Biol. Chem. published online March 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010016200

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