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Identification of Detergent-resistant Plasma Membrane Microdomains in Dictyostelium: Enrichment of Signal Transduction Proteins

Zhan Xiao and Peter N. Devreotes*

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2185

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Unlike most other cellular proteins, the chemoattractant receptor, cAR1, of *Dictyostelium* is resistant to extraction by the zwitterionic detergent, CHAPS. We exploited this property to isolate a subcellular fraction highly enriched in cAR1 by flotation of CHAPS lysates of cells in sucrose density gradients. Immunogold electron microscopy studies revealed a homogeneous preparation of membrane bilayer sheets. This preparation, designated CHAPS-insoluble floating fraction (CHIFF), also contained a defined set of 20 other proteins and a single uncharged lipid. Cell surface biotinylation and preembedding immunoelectron microscopy both confirmed the plasma membrane origin of this preparation. The cell surface phosphodiesterase (PDE) and a downstream effector of cAR1, adenylate cyclase (ACA), were specifically localized in these structures, whereas the cell adhesion molecule gp80, most of the major cell surface membrane proteins, cytoskeletal components, the actin-binding integral membrane protein ponticulin, and G-protein α- and β-subunits were absent. Overall, CHIFF represents about 3–5% of cell externally exposed membrane proteins. All of these results indicate that CHIFF is derived from specialized microdomains of the plasma membrane. The method of isolation is analogous to that of caveolae. However, we were unable to detect distinct caveolae-like structures on the cell surface associated with cAR1, which showed a diffuse staining profile. The discovery of CHIFF facilitates the purification of cAR1 and related signaling proteins and the biochemical characterization of receptor-mediated processes such as G-protein activation and desensitization. It also has important implications for the "fluid mosaic" model of the plasma membrane structures.

INTRODUCTION

G-protein-coupled receptors (GPCRs)[1] are universal signal transducers that allow cells to rapidly respond to extracellular stimuli. The several hundred members of this superfamily of seven transmembrane domain proteins share many properties (Strader *et al.*, 1995). All have a similar topological structure and all presumably transduce extracellular signals by activating a set of peripherally associated G-proteins. Many of the receptors undergo robust agonist-induced serine and threonine phosphorylation. These modifications are correlated with alterations in the number or affinity of surface-binding sites or in the efficiency of receptor coupling to the G-proteins, processes that lead to desensitization of the agonist-mediated responses.

A set of well-characterized G-protein-coupled cAMP receptors (cARs) are found in *Dictyostelium*, a free-living amoeba. The cARs mediate cellular responses to extracellular cAMP and thereby program development in this organism. Soon after the developmental program is triggered by nutrient depriva-

* Corresponding author.

[1] Abbreviations used: cAR, cAMP-receptor; CHIFF, CHAPS-insoluble floating fraction; DIG, detergent-insoluble glycosphingolipid-enriched membrane domain; GPCRs, G-protein-coupled receptors; GPI, glycosyl-phosphatidylinositol; PC, phosphotidylcholine; PE, phosphotidylethanolamine; TIC, Triton-insoluble complex; TIFF, Triton-insoluble floating fraction.

tion, the amoebae spontaneously aggregate: Central cells secrete cAMP at 6-min intervals and surrounding cells, sensing the nucleotide, respond by advancing chemotactically toward the center and by secreting additional cAMP, thereby relaying the signal distally. As the multicellular structure formed by the aggregating cells undergoes morphogenesis, eventually forming a fruiting body, cells in the anterior and posterior, under the continued influence of cAMP, differentiate into stalk or spore cells (for review, see Parent and Devreotes, 1996).

Functionally, the cARs most closely resemble chemokine receptors, the receptors that mediate the chemotactic responses of mammalian phagocytic cells, such as neutrophils. The properties of chemotaxis and the spectrum of the biochemical responses triggered by chemoattractants are remarkably similar in these evolutionarily distant cell types, and both cell types use the components of a G-protein-linked system for signal transduction. Chemotaxis is a fundamental cellular response and many more of its features are expected to be universally conserved. These features may include the subcellular distribution of the chemoattractant receptors and associated signaling components.

Although extensive studies have elucidated many of the biochemical properties of GPCRs, their cellular organization is relatively less well characterized. It is typically assumed that these molecules are uniformly displayed on the cell surface. Some evidence has suggested, however, that they may be organized into specialized regions. For instance, there have been recent reports that some GPCRs are preferentially localized in caveolae (Anderson, 1993; Lisanti *et al.*, 1994) or other related structures of endothelial cells, such as Triton-insoluble floating fraction (TIFF; Hoessli and Rungger-Bradle, 1985), Triton-insoluble complex (TIC; Brown and Rose, 1992), or detergent-insoluble glycosphingolipid-enriched membrane domains (DIG; Parton and Simons, 1995). The distribution of cell surface receptors into such specialized compartments might control their accessibility to ligands or G-proteins and thus may be an important regulatory mechanism. To further investigate the distribution of the GPCRs before and after agonist-induced phosphorylation, we have focused on the cARs of *Dictyostelium*.

Previous attempts to solubilize cAR1, the predominant cAR present in early development, have revealed a differential sensitivity to extraction by various detergents. Specifically, the zwitterionic detergent, CHAPS, was found to solubilize most of the cellular membrane proteins while leaving cAR1 essentially unextracted (Hereld *et al.*, 1994). This observation suggested to us that these chemoattractant receptors are localized in specialized CHAPS-resistant subdomains of the plasma membrane. We discovered that these domains can be isolated by flotation in sucrose density gradients and therefore designated them CHAPS-insoluble floating fraction (CHIFF). In its detergent resistance, CHIFF resembles caveolae or other related structures such as TIFF, TIC, or DIG.

In addition to cAR1, these structures contain a defined set of approximately 20 proteins, including several elements of the cAR1 signaling pathway, and a single lipid that appears to be a sterol. Thus, they differ markedly in structure, protein, and lipid composition from the bulk plasma membrane. We speculate that CHIFFs are isolated microdomains of the plasma membrane, devoid of cytoskeleton. If so, our findings imply that the plasma membrane is not continuous and uniform but may be a mosaic of distinct domains containing specific sets of proteins and lipids.

MATERIALS AND METHODS

Cells and Cell Culture

Wild-type (AX-3) or car1$^-$/car3$^-$ cell lines (Caterina *et al.*, 1994) were grown in shaking cultures of HL5 (200 rpm/min at 22°). Transformed cell lines overexpressing cAR1 were maintained on Petri dishes in HL5 with G418 (20 μg/ml, Sigma, St. Louis, MO). Unless otherwise indicated, cells were washed off plates into shaking culture and grown until reaching a density of about 5×10^6/ml. Cells were washed once in DB (10 mM sodium phosphate, pH 6.2, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$), and cell development was carried out in shaking suspension (120 rpm at 22°) at a density of 2×10^7/ml. Cells were repeatedly stimulated with 50 nM cAMP at 6-min intervals during the period from 1 to 6 h following starvation (Devreotes *et al.*, 1987).

Preparation of Cell Lysate

Six-hour stage cells were centrifuged and washed once in TEB (40 mM Tris-HCl, pH 8.0, 2 mM EDTA, 50 mM NaCl), centrifuged again, resuspended at a density of 2×10^8/ml in ice-cold TEB plus protease inhibitors (TEBP): leupeptin (10 μM), pepstatin A (2 μg/ml), benzamidine (0.5 mM), and phenylmethylsulfonyl fluoride (1 mM). CHAPS powder (Sigma) was added at 20 mg/ml of cell suspension. The detergent was mixed with the cells and the mixture was held on ice for 5 min. For the initial CHAPS solubilization test, the lysate was centrifuged in a Sorvall SS34 rotor at 11,000 rpm for 15 min to separate the pellet from the supernatant fraction. The pellet was washed once and resuspended in TEBP to the starting volume. Lubrol PX was then added to the pellet suspension to 1%, and solubilization was carried out for 30 min at 4°C. After microfuge centrifugation for 15 min or centrifugation at $100,000 \times g$ for 60 min, the sample was divided into final pellet and supernatant fractions. Equal fractions of supernatant and pellet were analyzed on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The cAR1 distribution profile was determined by blotting with cAR1 antibody (serum R4; Klein *et al.*, 1985).

Construction of Sucrose Density Gradient and Ultracentrifugation

Sucrose crystals (Sigma) were added to the CHAPS lysate to a final concentration of 55% (wt/vol). A 8-ml linear 20–45% sucrose gradient (in TEBP) was formed over 4 ml of this sucrose-containing CHAPS lysate. The gradient was centrifuged at 36,000 rpm for 14 to 16 h in a Beckman SW41 rotor at 8°C. A thin white band in the middle section of the gradient was collected, diluted fivefold with TEB, and centrifuged in a SS34 rotor at 12,000 rpm for 15 min. This pellet preparation was designated CHIFF. In some cases, the gradi-

ent was pumped from the bottom into 12–15 fractions (~0.8 ml/fraction) and the fractions were analyzed by immunoblot. In later experiments, we found out that by replacing the linear gradient with a step gradient of 4 ml of 20% and 4 ml of 45% sucrose, we could obtain essentially the same CHIFF preparation.

Immunogold Electron Microscopy

The collected CHIFF was resuspended in TEBP at a density of 2×10^9 cell equivalents (ceq)/ml. Bovine serum albumin (BSA) and NaCl were added to 1 mg/ml and 150 mM, respectively. Preabsorbed cAR1 antiserum was added at a 1:200 dilution. Mixing was carried out for 1 to 2 h at 4°C. After three washes in TEB plus NaCl and BSA, the sample was combined with 1:25 or 1:4 (for saturation staining) diluted gold-conjugated secondary antibody (10 nm, Amersham, Arlington Heights, IL) in TEB plus NaCl and BSA and incubated at room temperature for 1 to 2 h. Five washes were carried out in TEB. The final pellet was fixed with 2% glutaraldehyde (22°C for 30 min), postfixed with OsO_4 (30 min on ice), and stained with uranyl acetate and lead citrate. Thin sections were cut and examined under a Philips 410 transmission electron microscope. Control samples were prepared in parallel with preimmune serum to ensure that the observed gold labeling was specifically dependent on the interaction of cAR1 with anti-cAR1 antibodies.

For whole-cell immunogold labeling, cells were plated onto 35-mm Petri dishes and then fixed/permeabilized with 2% glutaraldehyde in 50 mM cacodylate buffer containing 1% CHAPS for 5 min. Inclusion of CHAPS was necessary to permeabilize the cells in order for antibody molecules to access the C terminus of cAR1. Cells were then postfixed in 0.5% glutaraldehyde for 10 to 15 min. The fixed cells were washed twice for 15 min with 1 mg/ml $NaBH_4$ in water to quench glutaraldehyde and then blocked for 30 min with 3% BSA in wash buffer. Affinity-purified cAR1 antibody (1:200 dilution) was added and incubated for 1 h. After three 5-min washes with wash buffer, 1:20 diluted secondary antibody-gold conjugate (5 nm, Amersham) was added and further incubated for 1 h. After six 5-min washes, cells were further fixed with 1% OsO_4. Samples were then dehydrated, embedded in Eponate resin (Ted Pella), sectioned, and poststained with 1% uranyl acetate.

Preembedding Immunoperoxidase Transmission Electron Microscopy

cAR1-expressing cells were plated on 35-mm culture dishes (80% confluency). Two different solutions were used to prefix/permeabilize the cells for 1.5 min: 0.25% CHAPS/1% glutaraldehyde in phosphate-buffered saline (PBS) or 0.1% Triton X-100/1% glutaraldehyde in PBS. Pilot tests had been carried out using ^{125}I-labeled protein A to confirm that both of these conditions would render the cell interior accessible to labeling while leaving the cell morphology essentially intact. Cells were then postfixed with 0.5% glutaraldehyde in PBS for 10 min. Unreacted glutaraldehyde was quenched with two 15-min incubations with ice-cold 1 mg/ml sodium borohydride in PBS. The dish was blocked with 3% BSA/wash buffer for 1 h and then incubated with affinity-purified cAR1 antibody diluted 1:300 in 1% BSA/wash buffer for 1 h. After three washes (10 min each), goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Amersham) diluted 1:200 in 1% BSA/wash buffer was added and allowed to incubate for 1 h. After three 10-min washes, 0.5 mg/ml 3,3′-diaminobenzidine (Sigma) in 25 mM Tris-HCl (0.01% H_2O_2), pH 7.6, was added to the dish. Multiple dishes were used to test different reaction times. Cells were checked under a microscope for the brown product formation. To stop the reaction, each plate was rinsed twice for 10 min with Tris-HCl buffer (pH 7.5) and once with 0.1 M cacodylate (pH 7.4). Plates were then fixed with 2% glutaraldehyde and postfixed with 1% KFeCN-reduced OsO_4. After rinsing with distilled water, graduated dehydration was performed. Samples were embedded, and sections were cut and checked under the transmission electron microscope without poststaining. Appropriate control labeling tests were included in which cAR1-null cells were used or the primary antibody incubation step was omitted. In both cases no detectable labeling was found.

Immunoblotting

The CHIFF preparative gradients were fractionated into 12–15 fractions, and each fraction was solubilized in sample buffer and separated by SDS-PAGE (10% low bisacrylamide; Klein *et al.*, 1985). After transfer onto PVDF P membranes (Millipore), the blots were probed with different antibodies at appropriate dilutions. The sera for mPDE and gp80 were kindly provided by Richard Kessin and Chi-Hung Siu, respectively. The bound antibodies were visualized with secondary anti-rabbit antibody-HRP conjugates (New England Nuclear, Boston, MA) according to the manufacturer's recommendations. When the same blot was to be probed with a different antibody, stripping was performed at 55°C for 30 min with stripping solution (62.5 mM Tris, pH 6.5, 2% SDS, 2 mM 2-mercaptoethanol). Efficacy of stripping was confirmed by developing the blot with enhanced chemiluminescence reagents.

Concanavalin A (Con A) Overlays

The samples were resolved by 10% SDS-polyacrylamide gels and transferred onto a PVDF membrane. The membrane was blocked with 3% BSA in wash buffer for 1 h. Biotinylated Con A (Pierce, Rockford, IL) at 0.1 μg/ml in wash buffer was incubated with the membrane for 1 h. After three 10-min washes with TBS/T (2.45 g of Tris base, 8 g of NaCl, pH adjusted to 7.6, 1 ml of Tween 20/l), the membrane was further incubated with streptavidin-HRP conjugate (Pierce) at 0.1 μg/ml in 1% BSA-containing TBS/T for 1 h. The final visualization of Con A-binding proteins was by enhanced chemiluminescence (New England Nuclear). Specificity of the detection was confirmed by carrying out the initial incubation step with biotinylated-Con A supplemented with 200 mM α-methyl-D-mannoside (Sigma).

Cell Surface Labeling

Cell surface proteins were labeled with sulfo-*N*-hydroxysufosuccinimide (SNHS)-biotin (Pierce) using a modified Goodloe-Holland and Luna method (Goodloe-Holland and Luna, 1987). Briefly, 6-h suspension-developed AX3 cells were pelleted and resuspended to 4×10^8 cells/ml in DB. SNHS-biotin (0.2 mg/ml) was added to the suspension and cells were shaken at 150 rpm/min for 30 min at 4°C. The cells were washed once with DB and then twice with TEB (the 40 mM Tris in TEB also serves to quench unreacted SNHS-biotin). The cells were lysed with CHAPS and CHIFF was prepared from the lysate.

Differential Sensitivities of CHIFF Proteins to Various Extraction Conditions

CHIFF prepared from SNHS-biotin-labeled or unlabeled cells were resuspended in TEB to a density of 5×10^8 ceq/ml. Various detergents were added to the suspension, and extractions were carried out at 4°C with mixing for 2 h. Alternatively, the CHIFF pellet was directly resuspended in 0.2 M sodium carbonate buffer (pH 11.5). The detergents used were lubrol PX at 1% (wt/vol), Nonidet P-40 (NP-40) at 1%, deoxycholate at 1%, digitonin at 1%, CHAPS at 1.5%, octylglucoside at 2%, and dodecylmaltoside at 1%. Except for octylglucoside and dodecylmaltoside, which were purchased from Anatrace (Maumee, OH), all detergents were from Sigma. Soluble fractions were separated from insoluble fractions by 30-min centrifugation at 100,000 × *g*. Equal fractions of supernatant and pellet were loaded on SDS-polyacrylamide gels. After transfer onto PVDF membrane, proteins were visualized by either direct incubation with streptavidin-HRP (SNHS-biotin-labeled CHIFF) or Con A-biotin followed by streptavidin-HRP incubations (unlabeled CHIFF).

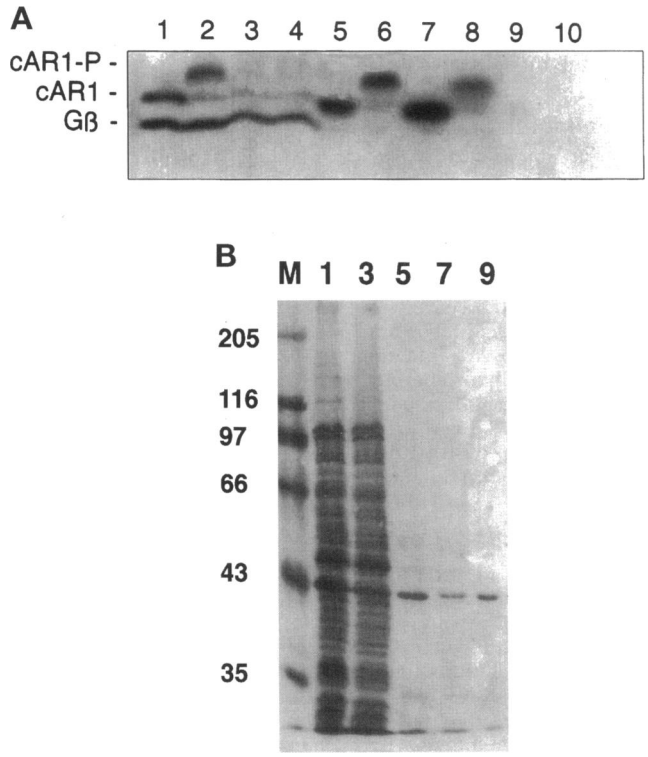

Figure 1. CHAPS extraction profile of intact cells. cAR1-expressing cells were treated with either 2 mM caffeine to maintain the basal state or 10 μM cAMP to induce cAR1 phosphorylation just prior to harvesting. CHAPS lysates and subcellular fractions were prepared and analyzed by immunoblots as described in MATERIALS AND METHODS. (A) Odd-numbered lanes represent samples prepared from basal cells; even-numbered lanes represent samples from cAMP-stimulated cells. Lanes 1 and 2, whole cells; Lanes 3 and 4, CHAPS-soluble extract; Lanes 5 and 6, CHAPS-insoluble pellet; Lanes 7 and 8, lubrol-soluble extract of CHAPS-insoluble pellet; Lanes 9 and 10, lubrol-insoluble pellet of CHAPS-insoluble pellet. cAR1 and Gβ were detected as described in MATERIALS AND METHODS. (B) The overall protein distribution profile was examined using Coomassie blue staining. Only fractions from unstimulated cells (odd-numbered lanes) were examined.

Lipid Composition Determination

Whole-cell crude membrane lipids (as control) or CHIFF lipids were prepared according to the Bligh-Dyer method (Bligh-Dyer, 1959). In addition, the procedure was modified to better extract polar or charged lipid species by using HCl-acidified methanol (0.6 ml 37% HCl in 50 ml methanol) instead of methanol alone. After drying down under a stream of nitrogen, the lipids were resuspended in small volumes of chloroform and run on silica gel HL (250 μm) TLC plates. Two different solvent systems were used to resolve the samples: system 1 (phospholipid plate): $CHCl_3$:MeOH:30% NH_3: dH_2O (60:40:5:2, vol/vol) and system 2 (neutral lipid plate): 0.5% NaCl:MeOH:NH_3 (50:50:1, vol/vol). Primary visualization was accomplished by spraying iodine vapor over the plates; final visualization was by spraying plates with 40% sulfuric acid and heating at 150°C for 10 min. The indicated lipid markers were also run in parallel on the same plates. Migration distance was measured for each lipid species and the respective RF value was calculated.

RESULTS

cAR1 Is Insoluble in CHAPS

As noted above, cAR1 is highly enriched in the pellet fraction of CHAPS extracts. To quantitate the recovery of cAR1 during this extraction process, we carried out the fractionation procedure described in MATERIALS AND METHODS. As illustrated in Figure 1A, cells were pretreated with (lane 2) or without (lane 1) cAMP to induce phosphorylation of the receptor. This phosphorylation can be visualized as a mobility shift on SDS-PAGE (Hereld *et al.*, 1994). Cells were extracted with 1.5% CHAPS, and the lysates were centrifuged at 13,000 × *g* for 15 min. The cAR1 immunoblot profile shows that essentially all of the cAR1 protein fractionated into the CHAPS-insoluble pellet fraction (lanes 5 and 6) and none remained in the supernatant fraction (lanes 3 and 4). Most of the cAR1 in this fraction can be solubilized by 1% lubrol PX (compare lanes 7 and 8 with lanes 9 and 10), indicating that the insolubility of cAR1 in CHAPS is not caused by nonspecific protein aggregation or denaturation. The same blot was reprobed with antiserum against the G-protein β-subunit (Gβ; Lilly *et al.*, 1993) to show that Gβ was completely solubilized by CHAPS (lanes 3 and 4) and none remained in the pellet (lanes 5 and 6). The Coomassie blue staining profile of the same fractions (Figure 1B) demonstrates that the vast majority of the cellular proteins are solubilized by CHAPS and are separated from the cAR1-enriched pellet (compare lanes 1 and 3). Cytoskeleton components, mainly actin, apparently cosedimented with the CHAPS-insoluble pellet and constituted a major portion of the protein in this fraction (lane 5).

CHAPS-insoluble cAR1 Is Associated with a Low-Density Fraction

The behavior of cAR1 in CHAPS suggests that it resides in a specialized cellular compartment. Since cAR1 is obviously an integral membrane protein, it is possible that an avid association with a unique lipid bilayer imparts the detergent-resistant property. To examine this possibility, we investigated the density of this insoluble fraction by equilibrium sucrose density centrifugation. We reasoned that this method might separate a light plasma membrane subdomain from other CHAPS-resistant materials including nuclear and mitochondrial remnants and the cytoskeleton. The soluble proteins and cytoskeleton components are expected to either pellet or remain confined to the bottom, heavier fractions. This would greatly facilitate the purification of this cAR1-enriched subcellular fraction. After lysis of the cells by CHAPS, sucrose was added to a final concentration of 55%, and a 45–20% linear sucrose gradient was formed over this sample. After overnight centrifugation, a light floccu-

lent band was visible near the midpoint of the gradient. The contents of the gradient were fractionated and analyzed directly by SDS-PAGE. The Coomassie blue staining profile of the gradient (Figure 2A) reveals that most proteins remained in the bottom layers. The cAR1 immunoblot (Figure 2B) displays that all of the chemoattractant receptors banded tightly at a lower density. The position of cAR1 corresponds with that of the aforementioned light band. The density of this species, which we have referred to as CHIFF, is about 1.12 g/ml judging from its gradient position. *Dictyostelium* plasma membranes were reported to have a density of 1.16–1.17 g/ml (Goodloe-Holland and Luna, 1987).

As controls to demonstrate the unique properties of the CHAPS extract, we prepared lubrol PX and Triton X-100 extracts of cAR1-expressing cells. These detergents completely solubilized cAR1 (i.e., it does not pellet at 100,000 × *g* for 1 h). The extracts were loaded under matched 20–45% sucrose gradients and fractionated identically. In these detergents, cAR1 remained near the bottom of the gradients, as expected, along with other soluble proteins, demonstrating that solubilized cAR1 does not have an unusually low density (our unpublished data).

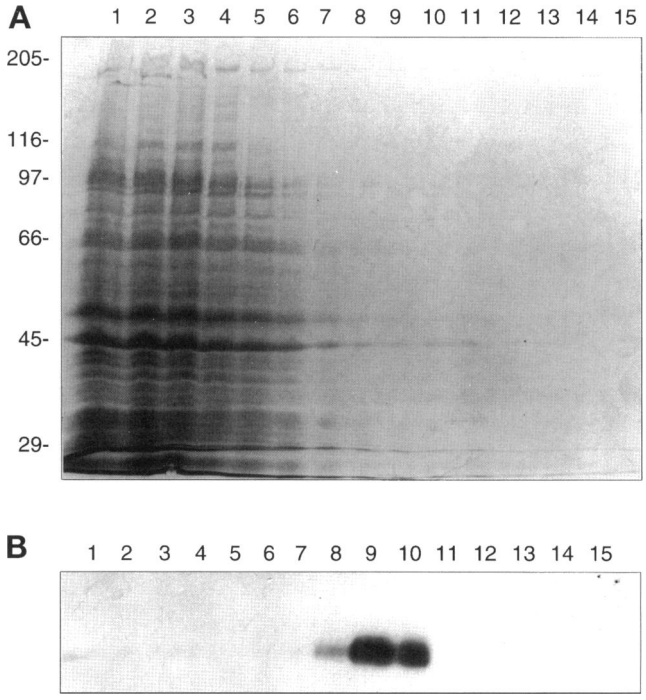

Figure 2. Fractionation profile of CHIFF preparative sucrose density gradient. CHAPS lysate of intact cells was analyzed on a linear sucrose density gradient as described in MATERIALS AND METHODS. The gradient (12 ml) was fractionated from bottom to top into 15 fractions (0.8 ml/fraction). Aliquots from each fraction were resolved by 10% low bis-SDS-PAGE and either directly stained with Coomassie blue (A) to reveal the overall protein profile or transferred to PVDF membranes and immunoblotted with cAR1 antiserum (B) to show cAR1 distribution. Lane 1 corresponds to the bottom fraction while lane 15 is the top fraction.

Fractionation Properties of Signal Transduction Components

To ascertain whether other components of the signal transduction pathway are also associated with the CHIFF fraction, similarly prepared gradients were stained with a variety of antisera. The membrane-bound phosphodiesterase mPDE (Lacombe *et al.*, 1986) is quantitatively localized in the same fractions as cAR1 as is apparent from its immunoblot profile (Figure 3A). A major portion of the aggregation-specific adenylate cyclase ACA (Pitt *et al.*, 1992), a downstream effector of cAR1, distributes to the same low-density fractions (Figure 3B). Taken together, the above results indicate that in addition to cAR1, two other critical membrane proteins of the cAR1 signaling pathway are primarily found in the same subcellular domain.

We also examined the distribution of several other surface membrane proteins. As shown in Figure 3, C and F, neither β- nor α-subunit of the heterotrimeric G-protein, G2, the G-protein functionally coupled to cAR1 (Kumagai *et al.*, 1989), substantially cofractionated with cAR1 on the sucrose density gradient. However, a minor fraction of Gβ was consistently found in the cAR1 fractions. Another G-protein, Gα8 (Wu *et al.*, 1994), which is not linked to the cAR1 signaling pathway, also fails to colocalize with cAR1 (Figure 3E). We also probed the same immunoblot with antiserum against gp80 (Muller and Gerisch, 1987; Kamboj *et al.*, 1989), a GPI-anchored membrane glycoprotein which mediates cell–cell adhesion during the early aggregation process. Its profile (Figure 3D) revealed that most of this protein fails to localize to the same fraction as cAR1, mPDE, and ACA. Ponticulin, an atypical integral membrane protein that mediates the attachment of actin to plasma membranes (Hitt *et al.*, 1994), is also absent from CHIFF (Luna, personal communications).

Protein Composition of CHIFF as Revealed by Silver Staining

CHIFF samples were silver stained to characterize their protein composition. To collect CHIFF and remove the contaminating soluble proteins trailing from the bottom of the gradient, the cAR1-containing fractions were pooled, diluted with excess buffer, and centrifuged. The pellet was washed once and resuspended in buffer. The silver-stained profile revealed that CHIFF contains about 20 different protein species. We numbered them into nine different groups with each containing one or multiple proteins according to their respective positions and intensities (Figure 4A, lanes 1 and 2, and Figure 4B). These groups were

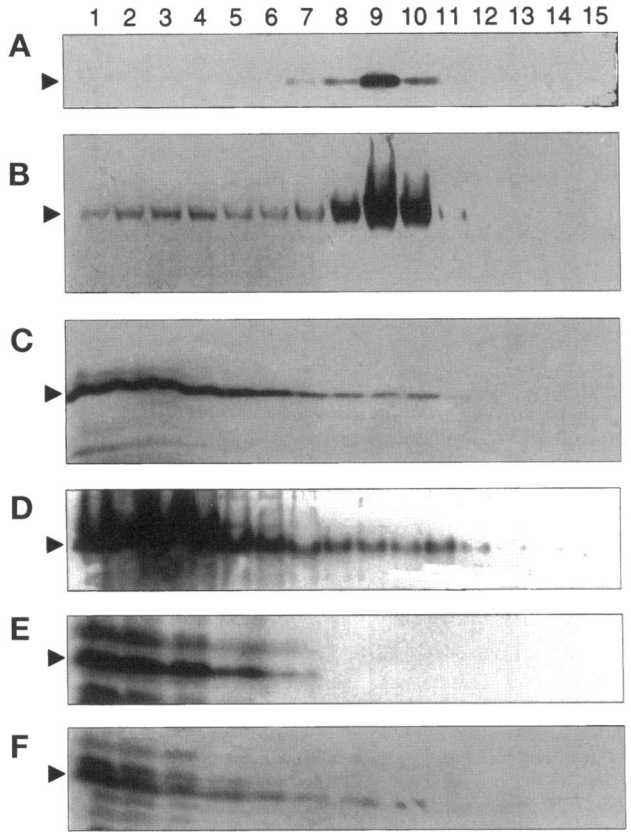

Figure 3. Fractionation profiles of other signal transduction components on the sucrose gradient. For A–D, the same immunoblotted filter as in Figure 2B was stripped and restained with each of the indicated antisera. For E and F, a similar gradient was run and fractionated, only that it was collected into 12 fractions and then probed with each of the indicated antisera. A, mPDE; B, ACA; C, Gβ; D, gp80; E, Gα8; and F, Gα2.

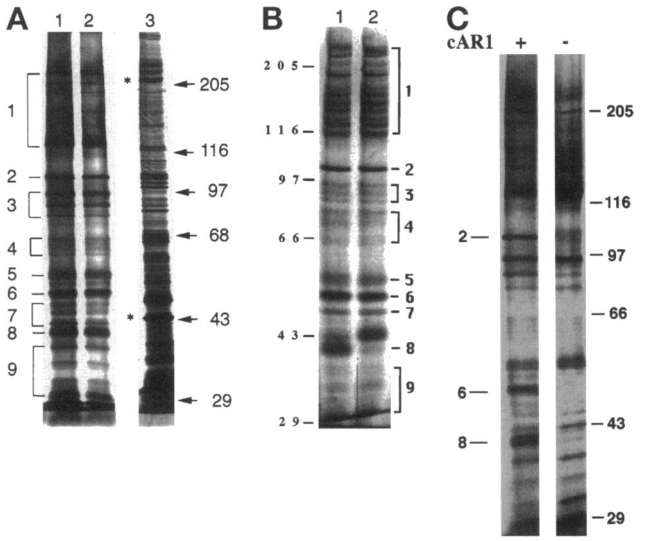

Figure 4. (A) Silver-staining profiles of CHIFF as compared with that of intact cells. CHIFF was prepared from cAR1-expressing cells using either the 20–45% step gradient method (lane 1) or 20–45% linear gradient method (lane 2). In comparison, intact cells were solubilized in SDS sample buffer and resolved on the same 10% low-bis gel (lane 3). In lanes 1 and 2, 2×10^8 ceq of CHIFF were loaded, whereas in lane 3, 4×10^5 whole cells were loaded. The major bands in CHIFF were numerically labeled into nine groups according to their migration positions and intensities. Actin and myosin bands in whole-cell samples are indicated by *. (B) Comparison of silver-staining profiles of CHIFFs prepared from basal and cAMP-stimulated cells. CHIFF was prepared from cAR1-overexpressing cells pretreated with either buffer (lane 1) or 10 μM cAMP. Bands in CHIFF were grouped according to A. Note that band 8 in lane 1 was shifted to the 43-kDa position in lane 2. (C) Comparison of silver-staining profiles of CHIFFs from cAR1-expressing and cAR1-null cells. CHIFFs were prepared from equal numbers of 6-h developed cAR1-expressing cells (cAR1+) or cAR1-null cells (cAR1−). The cell equivalent amount (2×10^8) was loaded for each sample. Bands 2, 6, and 8 (cAR1), which are present in cAR1+ but absent from the cAR1− cells, are indicated.

consistently observed in each preparation, although there were slight variations in the minor bands within the groups. The pattern of bands is totally different from that of intact cells (compare Figure 4A, lanes 1 and 2 versus lanes 3) or conventional plasma membrane preparations (our unpublished data), indicating that CHIFF represents a unique subfraction of the membrane. Notably, the cytoskeletal components actin and myosin (indicated by *), typically the most abundant plasma membrane associated proteins, are nearly absent. This is consistent with the previous observation that ponticulin is not found in CHIFF. CHIFF prepared by a 20–45% step gradient (lane 1) is almost identical to CHIFF prepared by the original linear 20–45% sucrose gradient method (lane 2), indicating that CHIFF is the only floating species in this density range.

We quantified the recovery of cellular proteins in CHIFF in several ways. Judging by relative silver-staining intensities, we estimated that CHIFF contains less than 0.2% of the cellular protein mass (lanes 1 and 2 have about the same amount of proteins as lane 3, but correspond to 500-fold more cells). This estimation was confirmed using the Bradford protein assay which indicated a 600-fold lower protein level in CHIFF versus intact cells. About 40–45 mg (wet weight) of CHIFF (containing about 1–2 mg of protein) are consistently recovered from 10^{10} cells (about 15 g wet weight and 1 g of protein). Marker proteins quantitatively enriched in CHIFF, such as cAR1, mPDE, and ACA, are purified about 500-fold. These results are summarized in Table 1. Since cAR1 is present at 10^5 copies/cell and constitutes about 5% of the CHIFF protein mass (estimation from Figure 4 by gel densitometry), we calculated that a single cell equivalent of CHIFF contains about 2×10^6 protein molecules.

The chemoattractant receptor cAR1 is one of the most abundant proteins in CHIFF (Figure 4A, band 8, lanes 1 and 2). cAR1 was identified as band 8 by

Table 1. Comparisons between cell and CHIFF

	Total wet weight (g)	Total protein (g)	Total cAR1 (μg)
10^{10} whole cells	15	1	100[a]
Equivalent CHIFF	0.045 (0.3%)	0.0015 (0.15%)	80[b] (80%)

The percentages in parentheses represent overall recovery rates of whole-cell components in CHIFF.

[a]cAR1 content of whole cells was calculated from total surface cAMP-binding sites and assuming one binding site per cAR1 molecule.

[b]cAR1 content of CHIFF was estimated by comparing the silver-staining intensity of cAR1 with that of a marker protein of known quantity.

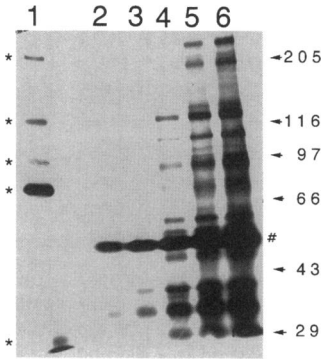

Figure 5. Surface-biotinylation labeling of CHIFF components. Intact cells were first surface labeled with sulfo-NHS-biotin and then lysed with CHAPS, and CHIFF was prepared. Labeled cells (5×10^6) were loaded in lane 1. Increasing amounts of CHIFF were loaded in lanes 2–6 (lane 2, 5×10^7 ceq; lane 3, 1×10^8 ceq; lane 4, 2.5×10^8 ceq; lane 5, 5×10^8 ceq, and lane 6, 1×10^9 ceq). After transfer onto PVDF membrane, labeled proteins were visualized by incubation with streptavidin-HRP conjugate (see MATERIALS AND METHODS). Major bands in labeled intact cells are denoted by *; the most predominant band in CHIFF (52-kDa band) is indicated by #.

several criteria. The position of band 8 corresponds to that of cAR1, and samples prepared from cAR1-null cell lines lacked band 8 (Figure 4C, lane 2). Furthermore, as illustrated above, stimulating intact cells with cAMP causes a reversible phosphorylation of cAR1 that can be visualized as a mobility shift on SDS-PAGE (Figure 1). Accordingly, CHIFF was prepared from a matched set of cAR1-overexpressing cells that had been treated with either buffer or cAMP. As demonstrated in Figure 4B, lanes 1 and 2, the 40-kDa band 8 in lane 1 was shifted to the 43-kDa position in lane 2, again confirming its identity as cAR1.

We speculate that most of the CHIFF proteins are uniquely localized in this fraction. This was already shown for cAR1, mPDE, and ACA. In addition, using an antiserum which recognizes the NH_2 terminus of band 6 to probe the initial preparative gradient, we noted that this protein is primarily localized in the CHIFF fraction (our unpublished observation). Further studies also indicate that CHIFF is a consistent structure, independent of the developmental conditions of the cell. A similar amount (wet weight and protein) of CHIFF is recovered from the growth phase and 6-h stage cells. Most of the proteins in CHIFF are expressed in both stages. A few of the proteins, such as bands 2, 6, and 8 (the cAR1 band), were only present in developed cells or cells constitutively expressing cAR1 and absent from cAR1-null cells (Figure 4C). In addition, mPDE and ACA are also developmentally regulated but not abundant enough to affect the staining pattern.

CHIFF Contains a Distinct Set of Cell Surface Proteins That Comprise About 5% of the Externally Exposed Proteins

To determine the exposed cell surface proteins residing in CHIFF, the fraction was prepared from intact cells labeled with sulfo-NHS, a highly hydrophilic and membrane impermeable reagent (Ingalls *et al.*, 1986). Whole cell and increasing amounts of the isolated CHIFF samples were run in parallel to characterize the percentage of cell surface that CHIFF comprises. As shown in Figure 5, lane 1, five proteins on cell surface are prominently labeled (indicated by *). The most heavily labeled protein has a molecular weight of around 84 kDa. A conventional plasma membrane preparation (Spudich and Spudich, 1982) prepared from these surface-labeled cells yielded essentially the same banding pattern, confirming that most of these proteins are plasma membrane proteins (our unpublished result). Actin and myosin, although very abundant in these cell and membrane preparations, were not labeled, indicating that no significant internal labeling occurred. The observed profile is not very different from that obtained previously (Ingalls *et al.*, 1986).

In contrast, the staining pattern of CHIFF is totally distinct, indicating that this cell surface structure is a unique subdomain of the plasma membrane (Figure 5, compare lanes 2–6 with lane 1). A 52-kDa protein was by far the most predominantly labeled band (indicated by #). Its position corresponds to that of band 6 in silver staining (Figure 4, A and B, lanes 1 and 2), in which it is also one of the most abundant species. With heavier loading (Figure 5, lanes 5 and 6), more bands become visible, these include a 120-kDa band, a 92-kDa band, a 38-kDa band, and a 32-kDa band. Since many of its composite bands are labeled, CHIFF is clearly derived from the cell surface. Labeling cells in the presence of permeabilizing reagents such as 0.1% Triton X-100 or 0.5% CHAPS, which exposed many internal proteins to the labeling (including actin and myosin), does not significantly alter the pattern of labeled bands found in purified CHIFF (our unpublished result), further indicating that few of the CHIFF proteins are internally derived. The overall pattern of SNHS-biotin labeling roughly resembles the silver-staining profile. Slight differences between these two

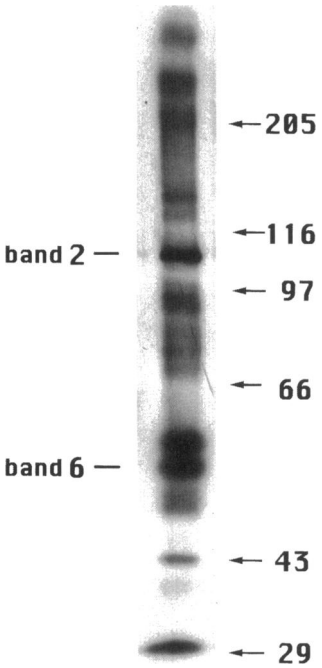

Figure 6. Con A overlay profile of CHIFF components: CHIFF sample was directly resolved on 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane. *N*-glycosylated proteins were visualized by incubations with Con A-biotin/streptavidin-HRP (see MATERIALS AND METHODS). Specificity of the overlay was confirmed by incubation with 200 mM α-methyl-D-mannoside. Bands corresponding to bands 2 and 6 of the previous silver staining are indicated for reference.

patterns can be explained by varying degrees of exposure and abundancies of lysine residues on different proteins. From comparison of the integrated intensities of the labeled bands between intact cell and CHIFF preparations, we estimated that CHIFF represents between 3 and 5% of the cell surface proteins.

CHIFF Proteins Are Mostly Glycosylated Integral Membrane Proteins

Since CHIFF is derived from the cell surface, most of its proteins should be glycosylated. To investigate this, we performed a Con A overlay test. As shown in Figure 6, about 15 CHIFF proteins were detected with varying intensities. Again, the overall pattern resembles the previous silver-staining profile with the most intense bands corresponding to band 6 and band 2 proteins. Column chromatography analysis of the lubrol-solubilized CHIFF proteins confirmed that they were primarily glycoproteins: Nearly all of the proteins observed by silver staining were quantitatively absorbed to a Con A column and subsequently released from the column by 50 mM α-methyl-D-mannoside. Among these were the mPDE, band 2, and band 6 (our unpublished result). These data show that most of CHIFF proteins are *N*-glycosylated and further documents the plasma membrane origin of CHIFF. A few proteins were not visibly labeled with Con A and did not absorb to the Con A column, the most prominent one among these is cAR1.

CHIFF was treated with different detergents and pH conditions in an attempt to characterize the solubility properties of its protein components. Because the Con A overlay profile of CHIFF closely reflects its overall protein composition, we used this technique to monitor the extraction results. Directly silver-stained or S-NHS-biotin-labeled samples yielded similar results (our unpublished result). As shown in Figure 7, 200 mM sodium carbonate buffer (pH 11.5) fails to extract most of the proteins (lanes 4), suggesting that there are few peripherally bound proteins. Among the nondenaturing detergents, 1% deoxycholate, 1% digitonin, and 1.5% CHAPS are very ineffective (lanes 3, 8, and 7, respectively), whereas 1% lubrol PX, 1% NP-40, 1% dodecylmaltoside, and 2% octylglucoside (samples 1, 2, 5, and 6, respectively) are able to solubilize most of CHIFF proteins. These properties further demonstrate that the CHIFF proteins are mostly integral membrane proteins and display differential sensitivities to various detergents.

Of the known CHIFF proteins, cAR1 and ACA are known to be integral membrane proteins. Furthermore, we microsequenced band 6 and cloned the gene encoding this protein. Hydropathy plot analysis indicates that it contains a membrane spanning domain (Xiao and Devreotes, unpublished data). The only known exception is mPDE, whose sequence does not contain any significant hydrophobic segments. As expected, this band appeared in the soluble extract of the sodium carbonate buffer treatment, pH 11.5 (our unpublished data). We presume that it is a peripheral protein binding to an integral membrane protein component of CHIFF.

Electron Microscopy of CHIFF

Preparations of CHIFF were analyzed by transmission electron microscopy (Figure 8). The fraction appears as homogeneous 100–200-nm-long membrane sheets or fragments. Upon closer inspection, these fragments often display a bilayer structure. To investigate the distribution of cAR1 on these membrane sheets, cAR1 immunohistochemistry was performed. When stained with purified cAR1 antibody and colloidal gold secondary antibody, the fragments were heavily decorated with gold particles (Figure 8, A and C). Control tests with preimmune serum show few gold particles (Figure 8B), confirming specificity of the staining. Careful inspection reveals that in some examples the gold label appeared only on one side of the membrane sheets (Figure 8C, arrowheads), consistent with our observation that this cAR1 antibody (R4) recognizes primarily the C-terminal region of the protein.

In Figure 8, A and C, not all CHIFF fragments were labeled. Although this might indicate that not all CHIFF contains cAR1, it could be simply due to insufficient primary or secondary antibody. To resolve this issue, we performed a second staining using excess reagents. In this trial, almost all of the fragments were

labeled with gold (Figure 8D), with the background only slightly increased (our unpublished data). Since cAR1 is quantitatively recovered in CHIFF and localized on essentially all of the particles, CHIFF is a very homogeneous preparation.

Immunoelectron Microscopy Study of cAR1 in Prefixed Cells

Based on the previous results, we use cAR1 as a CHIFF "marker" protein in an attempt to characterize its subcellular origin. CHAPS and NP-40 were used to provide access for the antibody to intracellular compartments. When added with glutaraldehyde, low concentrations of these detergents effectively permeabilized the cells without causing excessive damage to the cell shape as monitored by phase microscopy (see MATERIALS AND METHODS). We first carried out immunoperoxidase labeling of the fixed, permeabilized cells. As shown in Figure 9,A–D, cAR1 showed primarily peripheral membrane staining. Control staining of the same sample with only secondary antibody or staining of cAR1-null cells showed no labeling (our unpublished result), confirming specificity of the test. The pattern indicates that cAR1 is distributed over the whole cell surface and not confined to any highly enriched punctate regions (Figure 9, A–D). Under higher magnification (Figure 9, C and D), the labeled areas showed some uneven nature in staining intensities. The edge of the stained cell resembled a twisted ribbon (arrowheads) with the strongest labeling occurring at the twists of the ribbon, whereas generally the extended part of the ribbon showed the weakest staining. We speculate that the ribbons represent the undulating membrane surface stained and visualized through the entire thickness of the section. When the section runs directly through the membrane, a dark twist of ribbon is produced; when the section crosses the membrane at a low angle, the flat part of the ribbon is produced.

Some cells were completely extracted, but parts of the cell plasma membrane were retained as a string of sealed or semisealed vesicles outlining the cell contour. Some of these vesicles were strongly labeled and resembled the purified CHIFF (Figure 9, arrowheads). We speculate that these heavily labeled curved segments represent CHIFF forming from cell surface as the cell interior and membrane becomes extracted, and they were able to remain unextracted chiefly because of their detergent resistance.

We also used colloidal gold labeling to detect cAR1 (CHIFF) on cell surfaces (Figure 9E). The gentle fixation procedures previously used did not allow the secondary gold conjugate to penetrate the cells. Therefore, we used higher detergent concentrations (1% CHAPS); hence, the cells invariably appeared to be more extracted. Consistent with the immunoperoxidase staining results, the cells were extensively labeled at the peripheral membrane, although certain areas appeared to be more heavily labeled than others (Figure 9E, arrowhead).

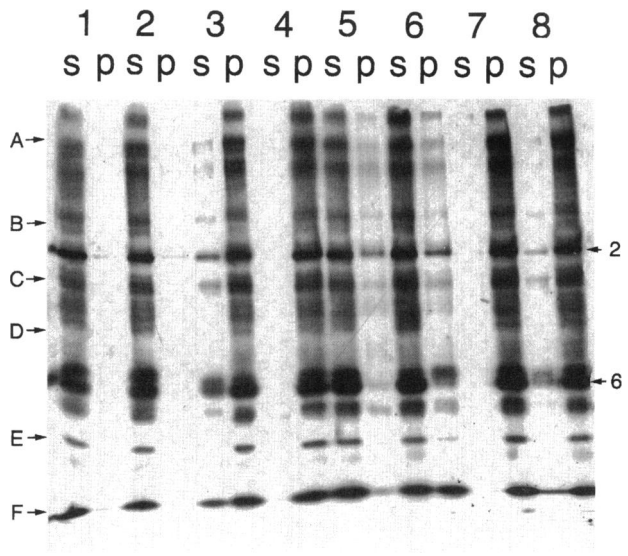

Figure 7. Differential sensitivities of CHIFF toward different extraction conditions CHIFF samples were treated with various detergents and pH conditions (see MATERIALS AND METHODS). The extracts were separated into soluble fractions (s) and pellet fractions (p) by centrifugation. The proteins in each fraction were visualized with the Con A overlay procedure (see Figure 6). Sample 1, 1% lubrol PX; sample 2, 1% NP-40; sample 3, 1% deoxycholate; sample 4, 200 mM sodium carbonate, pH 11.5; sample 5, 1% OG; sample 6, 1% DM; sample 7, 1% CHAPS; and sample 8, 1.5% digitonin. All percentage values are weight per volume. Letters on left-hand side denote positions of molecular weight markers: A, 205 kDa; B, 116 kDa; C, 97 kDa; D, 66 kDa; E, 43 kDa; and F, 29 kDa.

Unique Lipid Composition of CHIFF

The *Dictyostelium* cell plasma membrane primarily contains three species of lipids: phosphotidylcholine (PC), phosphotidylethanolamine (PE, including the plasmalogen form), and a sterol, with each species constituting about one- third of the lipid mass (Murray, 1982). In contrast, CHIFF seems to have only one major class of lipids, whose TLC profile is almost identical to that of δ-22-stigmasten-3β-ol which coruns with cholesterol (Table 2). This lipid is very poorly stained with iodine and can only be visualized after sulfuric acid charring of the plate. Tests are currently underway to determine this lipid. To control for the efficiency of the extraction procedure, the lipid contents of a crude plasma membrane preparation were obtained in the same way and analyzed on the same plates. The lipid composition of this sample was determined by comparison to marker lipids. Consistent with a previous study, three major lipids were found:

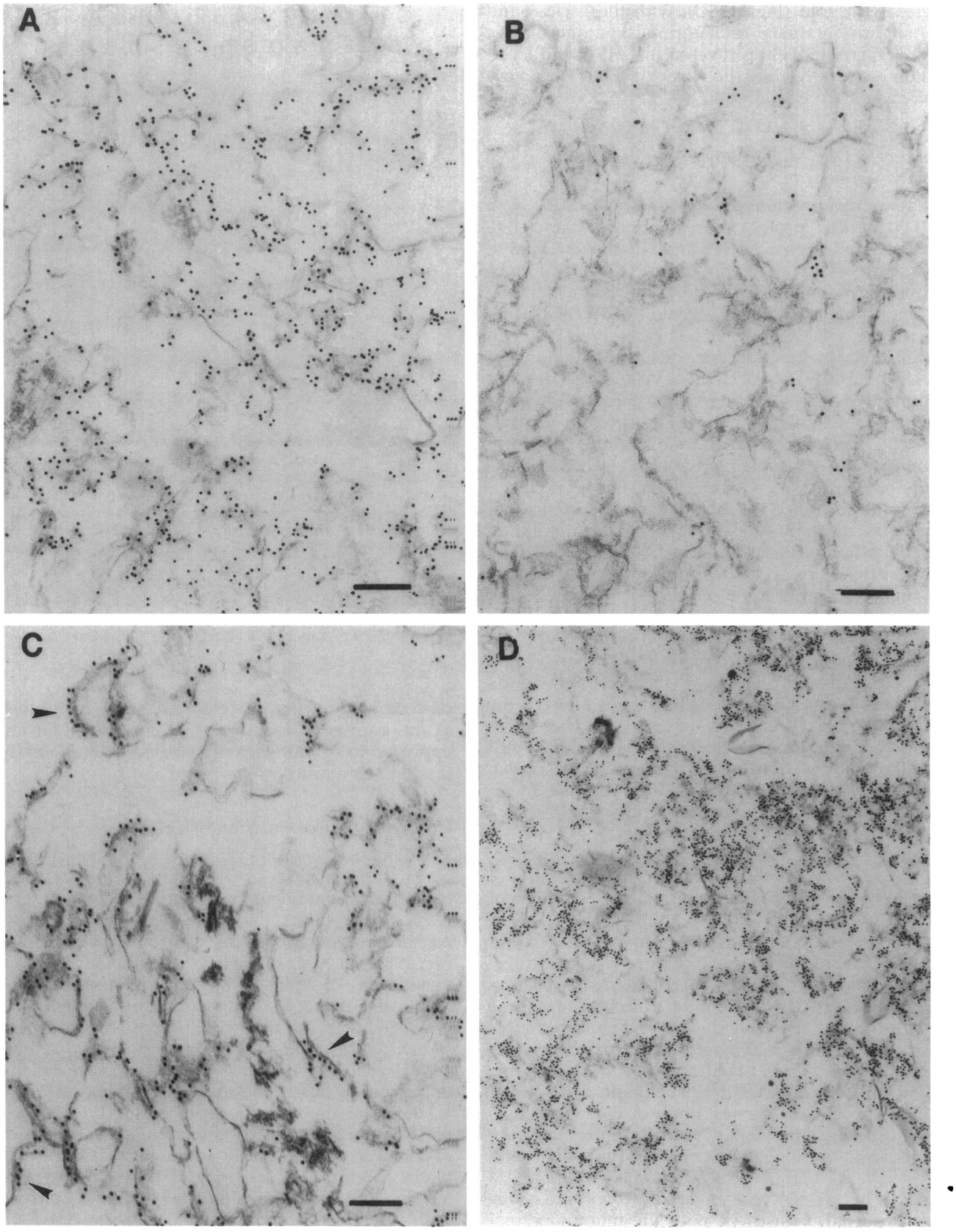

Figure 8.

PC, PE, and sterol (relative abundance ratio 2:2:3). The sterol spot in this sample cochromatographed with the CHIFF lipid, indicating that they might be the same species. The plasma membrane yields much more of this sterol than an equal cell equivalent amount of CHIFF, also suggesting that CHIFF contains only a small fraction of this membrane lipid. To minimize the chance of losing certain charged or polar lipids through the preparation process, a variant Bligh-Dyer extraction was performed in parrallel on the CHIFF sample in which acidified methanol (0.6 ml of 37% HCl added to 50 ml of methanol) was used in combination with chloroform to better extract these lipids. No difference in the final result was observed. CHAPS, which has an RF1 of less than 0.1, was not detected in any of these preparation.

DISCUSSION

We have isolated CHIFF, a novel plasma membrane-derived subcellular fraction, by virtue of its resistance to detergent extraction. Several lines of evidence indicate that CHIFF represents unique regions or reflects unusual properties of selected plasma membrane domains. First, its membrane protein composition is completely distinct from that of the plasma membrane: Surface protein labeling yields totally different profiles between conventional plasma membranes and CHIFF. The major labeled plasma membrane markers are mostly absent from CHIFF (Figure 5). The preparation also completely excludes gp80, the cell surface adhesion molecule. Although CHIFF contains less than 0.2% of the cellular proteins, it contains all of the cAR1, mPDE, most of ACA, and band 6. Second, saturation immunogold staining of CHIFF with cAR1 antiserum indicates that essentially all of the CHIFF fragments contain an abundant amount of cAR1, which is a minor plasma membrane protein. Often, only one side of these fragments are stained as would be expected from structures derived from plasma membranes. Third, CHIFF contains only one predominant lipid species whose chromatographic properties preclude it from being any of the common phospholipids.

We speculate that CHIFF represents microdomains of the plasma membrane with a distinct protein and lipid composition. Many of the proteins present in CHIFF are constitutively expressed: CHIFF prepared from growth stage cells or differentiated cells has a very similar profile. Also, the same amount of CHIFF can be obtained from vegetative and developed cells. These observations suggest that many of the proteins in CHIFF are structural proteins which may be required to create the necessary scaffolding for the various signal transduction components which become expressed as cells differentiate. Some of the CHIFF proteins may be minor components, such as ACA and mPDE, and may not substantially affect the staining profile.

The enrichment of signal transduction elements in the preparation suggests an organization of pathways or biological functions. We speculate that G-proteins and other related components are also associated with this region in the intact cell but poorly survive the CHAPS extraction. In fact, a minor fraction of Gβ was consistently present in CHIFF (Figure 3C). Different CHIFF preparation methods that utilize alternative detergents or no detergents at all should be developed. Interactions of G-proteins with the CHIFF domain may be more resistant to other fractionation procedures. Clustering of signal transduction elements in distinct regions might have several advantages: Little time and energy would be wasted in recruiting the necessary components and subtle differences in the composition of different clusters might confer specificity to the response. The juxtaposition of cAR1 with its downstream effector, ACA, and corresponding signal-attenuation element, PDE, should confer greater sensitivity and expedite the response process. In fact, this domain is not unlike a postsynaptic membrane which contains receptors, associated effectors, and enzymes that degrade neurotransmitters. It will be interesting to see whether other signal transduction systems mediated by GPCR are also organized in the same manner.

Some of the characteristics of CHIFF are reminiscent of caveolae, specialized surface membrane subdomains of endothelial cells. One of the properties of caveolae is the compartmentalization of signaling molecules, including GPCRs such as muscarinic acetylcholine receptor (Raposo *et al.*, 1987), β-adrenergic receptor (Raposo et al., 1989; von Zastrow and Kobilka, 1992), and endothelin receptors (Chun *et al.*, 1994). Purification of TIFF or TIC, which are believed to represent caveolae structures (Sargiacomo *et al.*, 1993), also involves the flotation of a detergent-resistant subcellular fraction in sucrose density gradients. In addition, cholesterol was found to be enriched in caveolae whereas our current data suggest that a cholesterol-like sterol is the primary lipid species in CHIFF. These similarities might indicate that CHIFF is a counterpart of mammalian caveolae. However, caveolae-like structures have not been reported in *Dic-*

Figure 8 (facing). cAR1 immunogold transmission electron microscopy (TEM) of CHIFF. CHIFF prepared from cAR1-overexpressing cells was incubated with preabsorbed cAR1-antiserum (A and C) or with crude preimmune serum (B). Secondary incubation was with 1:25 diluted colloidal gold-conjugated antirabbit antibody. Processing and fixation of the CHIFF were carried out as described in MATERIALS AND METHODS. In C, arrowheads are used to highlight the fragments stained unilaterally. In D, a 1:4 diluted colloidal gold reagent was used after the primary serum incubation. Bars, 200 nm.

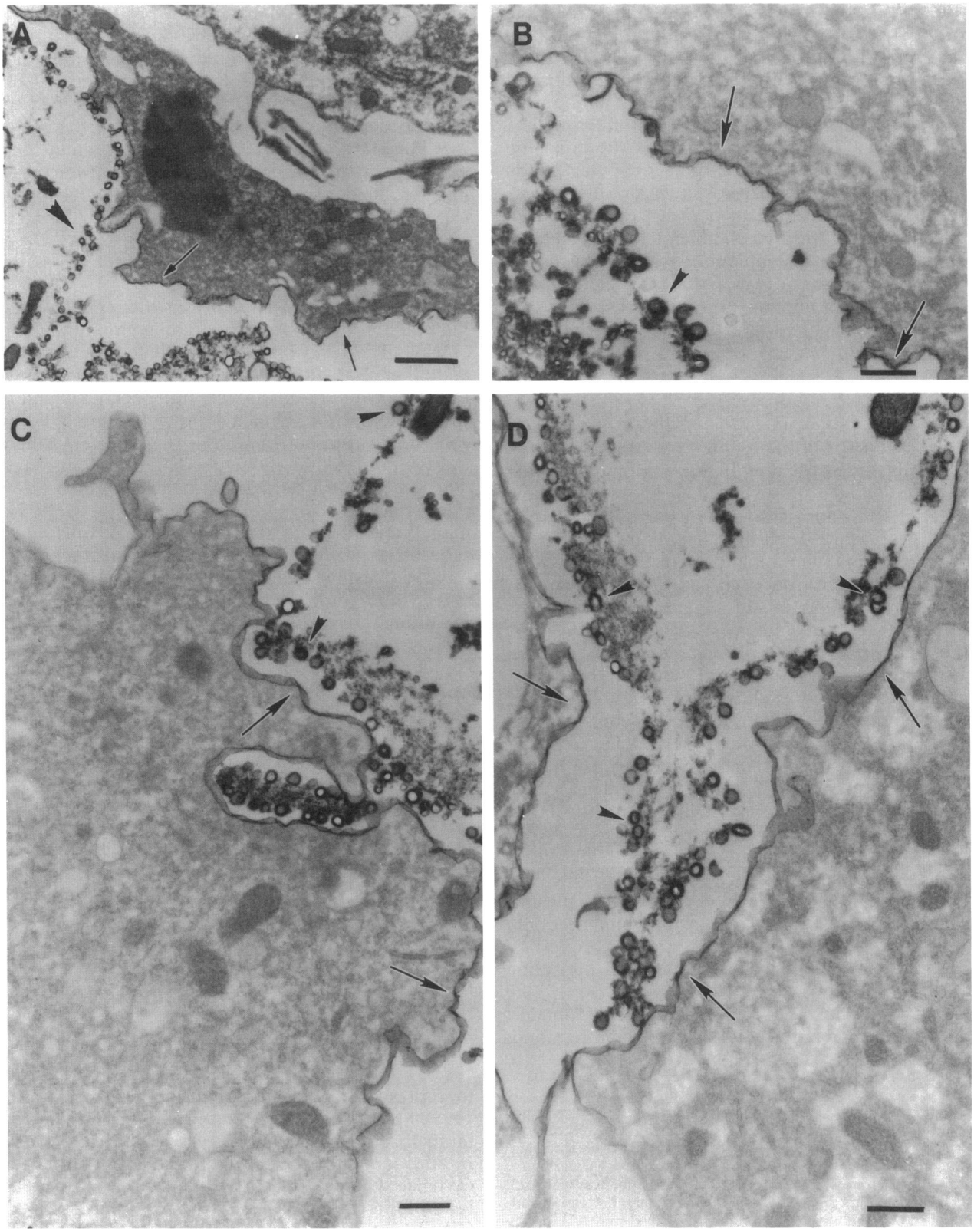

Figure 9. Immunoperoxidase and immunogold transmission electron microscopy of cAR1 on intact cells. cAR1 on whole cells was localized by two immunolabeling procedures: HRP-staining (A–D) and colloidal gold labeling (E). (A–D) Plated cells were fixed/permeabilized with

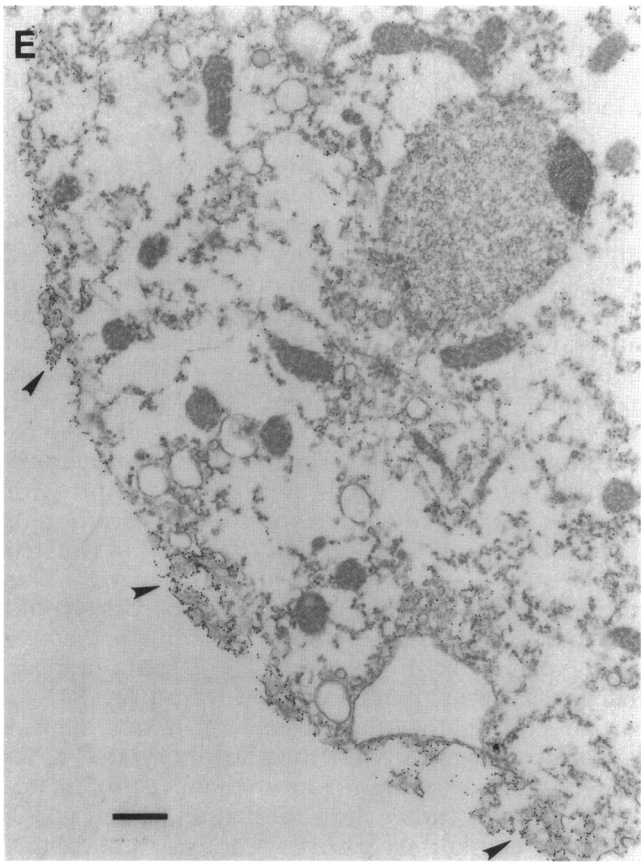

Figure 9 (cont). 0.25% CHAPS in 1% glutaraldehyde and processed for HRP staining as described in MATERIALS AND METHODS. Arrows indicate different cells (with varying magnifications) being labeled on the peripheral membranes. Also shown with arrowheads are several extensively extracted cells with variably stained surface vesicles (see text). Bars in B–D, 400 nm. Bar in A, 2 μm. (E) Plated cells were fixed/permeabilized with 1% CHAPS in 2% glutaraldehyde and processed for colloidal gold labeling as described in MATERIALS AND METHODS. Arrowheads indicate the areas more densely labeled than others. Bar, 600 nm.

tyostelium either by detection of a caveolin homologue or by electron microscopy. Moreover, there are several differences between these two presumptive specialized cell surface structures: First, the protein composition of caveolae appears to be more complex than that of CHIFF. For example, actin is a major protein in caveolae whereas it is nearly absent from CHIFF. Second, although caveolae contain a number of GPCRs, they are not nearly as enriched in caveolae as cAR1 is in CHIFF. Also, the downstream effector of cAR1, ACA, is primarily localized in the same CHIFF fraction; on the other hand, a similar case has not been reported for effector molecules of caveolae-enriched receptor proteins. Third, caveolae is specifically enriched for GPI-anchored proteins (Lisanti *et al.*, 1988; Brown and Rose, 1992); but gp80, one of the most prominent *Dictyostelium* GPI-linked proteins (Stadler *et al.*, 1989), is not localized to CHIFF (Figure 3).

Our biochemical results seem to be paradoxical with our morphological observations. The uniform size of CHIFF fragments, and the unidirectional orientation of cAR1 on these fragments suggest that they are derived from distinct structures on the cell surface. In contrast, electron microscopic studies of prefixed intact cells indicate that cAR1, a CHIFF marker protein, is diffusely distributed over the cell surface rather than confined to punctate domains. Fluorescent fusion protein studies using cAR1 tagged with green fluorescence protein (from the jelly fish *Aequorea victoria*) also showed that the receptor is uniformly distributed on cell surface membranes (Xiao, Zhang, Murphy, and Devreotes, unpublished data). We speculate that the cellular precursors of CHIFF are too small and closely apposed to be clearly resolved by HRP-labeling method (whose signals are produced by a diffusible enzyme reaction product), and it is only after CHAPS extraction that they coalesce to form the more visible CHIFF fragments (though the fusion has to take place in such an uniform way so that the asymmetry of cAR1 distribution is still preserved). This would predict that CHIFF does not directly correspond to in vivo structures. A similar hypothesis has been proposed to explain the homogeneous cell surface distribution profiles of certain TIFF or TIG-associated proteins such as GPI-linked membrane folate receptor α (Rijnboutt *et al.*, 1996).

This diffuse distribution profile of CHIFF seems inconsistent with the evidence indicating that CHIFF contains only a small fraction of the membrane proteins. It is unlikely that CHIFF represents a highly

Table 2. Mobility analysis of CHIFF lipid with silica gel TLC

Lipid	CHF	CL	SM	PE	PS	PI	PA	PC	SPH	DAG	LYS
RF1	0.75	0.75	0.1	0.3	<0.02	0.04	0.81	0.21	0.55	0.83	<0.02
RF2	0.35	0.36	<0.02	<0.02	<0.02	<0.02	0.53	<0.02	<0.02	0.53	<0.02

RF1, migration rate in first solvent system (phospholipid plate); RF2, migration rate in second solvent system (neutral lipid plate). See MATERIALS AND METHODS for solvent compositions. CHF, CHIFF; CL, cholesterol; SM, sphingomyelin; PS, phosphotidylserine; PI, phosphotidylinositol; PA, phosphotidic acid; SPH, sphingolipids; DAG, diacylglycerol; LYS, lysophosphotidic acid.

purified plasma membrane preparation devoid of peripheral membrane proteins and cytoskeletal components, since it contains minimal PC or PE and lacks most of the plasma membrane proteins. It is more likely that plasma membranes of cells are not uniformly organized. Some regions on the membrane may have a higher protein density and be enriched in PC and PE. These regions may be more sensitive to CHAPS extraction, and, hence, will fractionate in the bottom soluble fractions of the CHIFF preparative gradient. Alternatively, they could be CHAPS resistant but exist as a high-density species by virtue of association with the cytoskeletal components and will partition into the pellet fraction. CHIFF may correspond to other areas which are relatively protein poor, sterol rich, and quantitatively contain the specific set of proteins that we have isolated. This enhanced lipid:protein ratio is manifested as lower density in sucrose gradient flotation experiment and greatly expedites the biochemical purification of these domains.

Previous studies have indicated that the plasma membrane is organized into cholesterol-rich and cholesterol-poor regions (for review, see Liscum and Underwood, 1995). Thus, the plasma membrane can be viewed as a mosaic of fluid domains rather than a homogeneous fluid mosaic. Our findings are consistent with this view and further suggest that it should be extended to the protein components of the membrane. Sets of specialized membrane proteins such as those in CHIFF may be associated with specific lipids and remain isolated from the bulk proteins and lipids of the membrane.

Our preliminary results suggest that CHIFF contains a single cholesterol-like lipid that may be δ-22-stigmasten-3β-ol, the major sterol species in *Dictyostelium discoideum* (Murray, 1982). Interestingly, sterols have been found to be preferentially enriched in plasma membranes and in membranes of phagosomes as compared with endoplasmic reticulum, nuclear membrane, or mitochondrial membranes which contain virtually no sterols (Favard-Sereno *et al.*, 1981). This observation seems inconsistent with our electron microscopic findings showing that CHIFF assumes a membrane bilayer structure. It is possible that some additional lipids which may impart the bilayer-forming potential were lost during the preparation process. Highly polar lipids such as sulfated polyglycolipids and gangliosides may partition into the upper aqueous (methanol-water) phase during Bligh-Dyer extraction. We are currently investigating this possibility.

The isolation of CHIFF will allow us to determine whether or not receptor redistributes during persistent ligand stimulation. For many GPCRs, continuous or repeated exposure to high concentrations of ligand leads to an attenuated cellular response, usually termed desensitization or adaptation. The proposed mechanisms for desensitization of GPCRs include 1) receptor sequestration or internalization, 2) receptor loss, and 3) receptor phosphorylation which affects efficiency of coupling to G-proteins. We have previously shown that agonist occupancy induces a decrease in the affinity of cAR1 for cAMP coinciding with the phosphorylation of cAR1 (Caterina *et al.*, 1995a,b). Immunofluorescence studies suggested that the phosphorylated cAR1 remained at the cell surface even after prolonged stimulation (Caterina *et al.*, 1995a). Our current biochemical results are consistent: The phosphorylated form of cAR1, which corresponds to the agonist-desensitized state, still remains completely associated with the CHIFF fraction (Figure 4B) and hence still resides on the cell surface. This result further demonstrates that the induced decrease in affinity for cAMP is not due to either receptor sequestration or internalization. We have found that cAR1 in isolated CHIFF still retains its cAMP-binding capacity. This means that the CHIFF purification procedure could be used to prepare a large quantity of purified active cAR1 species. With purified CHIFF containing either the basal or phosphorylated form of cAR1, we can directly test the other possible scenarios, such as lower intrinsic affinity for the phosphorylated cAR1 or decreased coupling for its cognate G-proteins.

Preliminary studies indicate that cAR2 and cAR3, the other cell surface cAMP receptors in *Dictyostelium*, are also exclusively localized to CHIFF. Other GPCRs in *Dictyostelium*, such as folate receptor, may also be enriched in CHIFF. It has been possible to heterologously express several mammalian GPCRs, such as muscarinic receptor and β-adrenergic receptor, in *Dictyostelium* (Voith and Dingermann, 1995). We are currently assessing whether these receptors are targeted to the CHIFF compartment. This being the case, the ease of purifying CHIFF from large amounts of axenically grown *Dictyostelium* cells will greatly facilitate the biochemical purification and characterizations of these otherwise recalcitrant receptors.

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