# The βγ subunits of heterotrimeric G proteins acquire detergent insolubility directly at the plasma membrane

Armin Rehm, Hidde L. Ploegh*

*Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, 40 Ames Street, E17-322, Cambridge, MA 02139, USA*

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**Abstract The subunits of heterotrimeric G proteins, Gα and Gβγ, are found in association with detergent-resistant domains in most mammalian cell types, implicating such domains in G protein-coupled signaling. The pathway by which the βγ complexes are targeted to these detergent-resistant domains was unaffected by the brefeldin A-imposed block on endoplasmic reticulum-to-Golgi transport. We have used subcellular fractionation and β subunit-specific immunoprecipitation to localize the acquisition of detergent insolubility of newly synthesized βγ complexes. The β subunits cofractionate with plasma membranes, and acquire detergent insolubility coincident with arrival in the plasma membrane fractions. This association was not affected by phorbol 12-myristate 13-acetate-induced activation of Proetin kinase C.**



*Key words:* Heterotrimeric G protein βγ subunits; Monoclonal antibody; Plasma membrane purification; Detergent-resistant membrane; Heptahelical receptor; Protein kinase C

## 1. Introduction

Heterotrimeric guanine nucleotide binding proteins (G proteins) are involved in signal transduction at the plasma membrane but also in intracellular vesicular transport [1,2]. G protein-coupled heptahelical receptors are synthesized on membrane-bound ribosomes and are inserted into the endoplasmic reticulum (ER), where they are glycosylated cotranslationally and obtain their proper folding, including covalent modifications such as disulfide bonds. In contrast to the receptors, the subunits of heterotrimeric G proteins are synthesized on ribosomes in the cytosol, followed by subunit modifications such as myristoylation and palmitoylation of the α subunit and prenylation of the γ subunit. Both types of modifications, which presumably take place co- or posttranslationally in a cytosolic compartment, were shown to be a prerequisite for membrane anchoring of the G protein subunits [3,4].

Heterotrimeric G proteins occur in specialized domains; depending on the cell type such domains are referred to as either caveolae or detergent-resistant membranes (DRM) [5]. The latter are operationally defined by their insolubility in non-ionic detergents and by their low-buoyant density in sucrose density gradients [6]. DRM were proposed to play a role in sorting of GPI-anchored and viral proteins at the trans-Golgi network (TGN) for delivery to the apical surface [7]. Other studies proposed a role for DRM in potocytosis and in signal transduction, suggested by their enrichment for signaling molecules such as heterotrimeric G proteins, Src-family non-receptor-tyrosine kinases, H-Ras, CD45, CD4, and eNOS [5,8–10].

We have shown that βγ subunits of heterotrimeric G proteins associate with DRM in neuroblastoma cells with rapid kinetics [11]. The transport pathway taken by cytosol-derived βγ subunits to rapidly formed detergent-resistant domains was not affected by a block imposed on ER to Golgi trafficking, as brought about by brefeldin A (BFA) treatment. Based on this observation we proposed that cytosolic βγ subunits are inserted into DRM probably at the plasma membrane itself [11].

Here we demonstrate by subcellular fractionation and β subunit-specific immunoprecipitation that G protein βγ subunits acquire detergent insolubility directly at the plasma membrane, whereas a model heptahelical receptor remains detergent-soluble under identical conditions. We propose a model for differential targeting of heptahelical receptors and G protein subunits to specialized microdomains at the plasma membrane. We show that detergent insolubility of βγ complexes in neuroblastoma cells is not affected by activation of protein kinase C (PKC).

## 2. Materials and methods

### 2.1. Cell culture and metabolic labeling

The human neuroblastoma cell line IMR-32 was grown in DME medium, supplemented with 10% FCS, L-glutamine (2 mM), penicillin (1:1000 dilution U/ml), and streptomycin. CHO cells stably transfected with a human Flag-platelet-activating factor (PAF) receptor cDNA were grown in UltraCHO medium (Bio Whittaker) supplemented with 5% FCS, penicillin, streptomycin and 800 μg/ml geneticin (Gibco BRL) (transfected cells were kindly provided by Dr. N.P. Gerard, Harvard Medical School, Boston, MA) [12].

The PKC activator phorbol 12-myristate 13-acetate (PMA) and its inactive stereoisomer 4α-phorbol 12,13-didecanoate (PDD) were purchased from Calbiochem-Novabiochem.

Metabolic labeling of IMR-32 cells with [$^{35}$S]methionine/cysteine (80/20) (Express protein labeling mix, DuPont-NEN, Boston, MA) and pulse-chase experiments were performed as described [11].

CHO cells transfected with the human Flag-PAF receptor were metabolically labeled for 2 h, and cells were washed twice with phosphate buffered saline (PBS) and once with PAF binding buffer (0.15 M choline chloride, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 0.25% BSA) [12]. Cells were incubated in binding buffer at 4°C or 37°C in the presence of various concentrations of PAF (Sigma, St. Louis, MO). Ligand induction was stopped by washing the cells and addition of lysis buffer (see below).

*Corresponding author. Present address (of both authors): Harvard Medical School, Department of Pathology, 200 Longwood Ave., Boston, MA 02115, USA. Fax: (1) (617) 432-4775.
E-mail: ploegh@hms.harvard.edu

*Abbreviations:* G proteins, heterotrimeric guanine nucleotide binding proteins; DRM, detergent-resistant membranes; TGN, trans-Golgi network; ER, endoplasmic reticulum; BFA, brefeldin A; mAb, monoclonal antibody; PNS, postnuclear supernatant; TX-100, Triton X-100; Staph. A, *Staphylococcus aureus*; WGA, wheat germ agglutinin; PMA, phorbol 12-myristate 13-acetate; PAF, platelet-activating factor


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*2.2. Antibodies*

The β subunit-specific monoclonal antibody (mAb) ARC5 was raised as described [13]. This antibody recognizes β only when not associated with γ, as may be experimentally imposed by the presence of 0.2% SDS in the cell lysis buffer. The $Na^+/K^+$-ATPase, α subunit-specific antibody served as plasma membrane marker (kindly provided by Dr. W.J. Nelson, Stanford University, CA). Anti-Flag M5 antibody, recognizing the amino acid sequence MDYKDDDDKEF, was from Kodak.

*2.3. Gel electrophoresis and immunoblotting*

SDS-gel electrophoresis was performed as described, using 12.5% acrylamide gels. Radioactively labeled samples were visualized by fluorography using dimethylsulfoxide-2,5-diphenyloxazole and exposure to Kodak XAR-5 films. For immunoblotting, aliquots from each fraction were resolved on 12.5% acrylamide gels and blotted to nitrocellulose (0.45 μm pore size, Bio-Rad, Hercules, CA). Further analysis was as described [11].

*2.4. Subcellular fractionation*

Homogenization conditions were exactly as described [11].

Differential centrifugation of a postnuclear supernatant (PNS) was essentially as described [11], except that the first centrifugation was done at 16 000×*g* for 15 min.

To study the occurrence of detergent-resistant membrane domains in such fractions, we made use of our previously described method, using octylglucoside for re-extraction of detergent-resistant membranes [11]. Prior to immunoprecipitation the amount of radioactivity was normalized for individual chase points.

Immunoprecipitations for recovery of β subunits were done exactly as described [11].

*2.4.1. Discontinuous sucrose gradient.* Plasma membranes were further separated from microsomal membranes by a modification of a method described previously [14] (see Fig. 2B). The 16 000×*g* pellet (see above) was resuspended in 1.3 M sucrose in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (2 μg/ml), and 3 ml of sample was overlaid with 1.2 M (1.5 ml), 1.14 M (4 ml), 0.99 M (1.5 ml), and 0.9 M (1.5 ml) Tris-HCl buffered sucrose. The flotation gradient was centrifuged to equilibrium at 170 000×*g* for 16 h in a SW-41 rotor (Beckman Instruments, Palo Alto, CA) at 4°C. Fractions (1 ml) were taken from the top and processed as follows. Fractions were lysed directly in an equal volume of 2×lysis buffer, containing 2% TX-100, for 30 min on ice, followed by centrifugation at 14 000 rpm in an Eppendorf centrifuge. Detergent-insoluble domains were then re-extracted in the presence of 60 mM octylglucoside. Alternatively, fractions were diluted by addition of PBS, with the addition of 0.5 mM PMSF and aprotinin (2 μg/ml), and membranes were pelleted at 100 000×*g* for 1 h in a TLA-45 rotor, TL-100 ultracentrifuge, followed by differential detergent-extraction.

*2.4.2. Absorption of plasma membranes on wheat germ agglutinin (WGA) agarose.* In some experiments, fractions as obtained after centrifugation on sucrose gradients were divided into two equal portions, and membranes were pelleted as shown above (see Fig. 2B). One half of these membranes was first lysed in TX-100 containing lysis buffer, followed by re-extraction in octylglucoside. The corresponding half of the membranes was diluted in PBS (with 0.5 mM PMSF, aprotinin (2 μg/ml) added) and incubated instead with 400 μg/ml WGA agarose beads (Pharmacia Biotech, Piscataway, NJ) for 4 h at 4°C. Bound plasma membranes were separated from non-bound membranes by pelleting (1000×*g*, 5 min, 4°C). Membrane proteins were released from WGA agarose beads by lysis in octylglucoside containing lysis buffer, followed by immunoprecipitation.

*2.5. Organelle-specific membrane marker assays*

Mannosidase II activity (Golgi) was determined by hydrolysis of *p*-nitrophenyl-α-D-mannopyranoside (Sigma Chem. Co., St. Louis, MO) with volumes adapted for use in 96-well plates [14]. After incubation at 37°C for 1 h reactions were stopped by addition of 100 μl 0.5 M glycine/0.5 M $Na_2CO_3$, pH 10. Absorbances were detected in a fluorescence photometer at 405 nm. The distribution of the plasma membrane marker α-$Na^+/K^+$-ATPase was determined by immunoblot.

## 3. Results

The βγ subunit complexes of heterotrimeric G proteins acquire detergent insolubility shortly after synthesis. The BFA-imposed block of the anterograde ER-to-Golgi pathway does not affect the association of βγ subunits with detergent-resistant membranes, suggesting that cytosolic βγ subunits enter these domains at a site distal from the BFA block [11].

In which subcellular compartment does detergent insolubility of cytosol-derived βγ subunits occur? To resolve the intracellular location of βγ subunits we used a combination of pulse-chase experiment and subcellular fractionation. A postnuclear supernatant was fractionated by differential centrifugation into a plasma membrane-enriched fraction, microsomes and cytosol, followed by a differential extraction in TX-100- and octylglucoside-containing lysis buffers. This lysis protocol provided us with non-ionic detergent-resistant membranes, which exhibit essentially the same properties as those obtained as low-density protein-lipid complexes, separated by sucrose density gradient centrifugation [11].

The β subunit appeared in the Triton-extracted plasma membrane pool after 10 min of chase, whereas the maximum of βγ activity in this pool was obtained after 30 min of chase (Fig. 1). Re-extraction of the plasma membrane and light microsomal pool with octylglucoside revealed the existence of a non-ionic detergent-resistant pool of β subunits associated with plasma membranes, but not with lower density microsomal membranes. Consistent with our previous suggestion, this association with detergent-resistant membranes

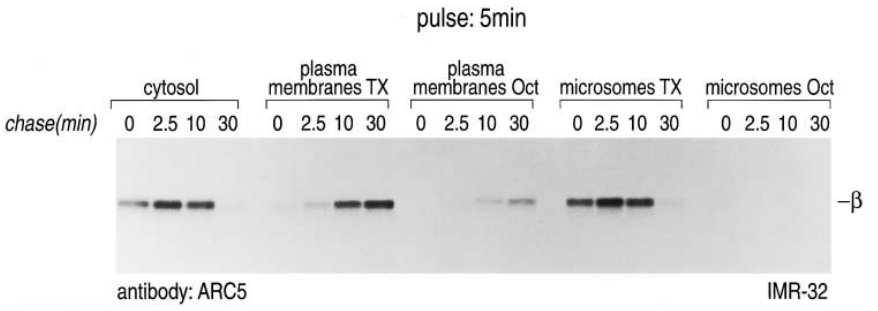


Fig. 1. Gβ is associated with non-ionic detergent-insoluble domains in the plasma membrane enriched pool. IMR-32 cells were pulse-labeled for 5 min with 500 μCi [$^{35}$S]methionine and chased for the designated times. Cells were homogenized, and membrane or cytosol fractions were prepared by differential centrifugation, yielding fractions designated plasma membranes (16 000×*g* pellet), microsomes (100 000×*g* pellet), cytosol (100 000×*g* supernatant). Membranes were first lysed in TX-100 (TX)-containing lysis buffer, followed by extraction of the detergent-insoluble pellet in octylglucoside (Oct). Prior to immunoprecipitation with the β subunit-specific mAb ARC5, the amounts of radioactivity for individual chase points were normalized for the soluble and pellet fractions. Samples were analyzed by 12.5% SDS-PAGE.

was not prevented by a BFA-imposed block of ER-to-Golgi trafficking (data not shown).

We used differential centrifugation in conjunction with subcellular fractionation on a discontinuous sucrose gradient and β subunit-specific immunoprecipitation to obtain a more precise subcellular localization of βγ subunit insolubility (Fig. 2A), as outlined in the flow sheet (Fig. 2B). The vast majority of β subunits after Triton extraction were recovered at the characteristic flotation density of the plasma membranes [14]. We recovered little material from the dense region of the gradient (fractions 7–10) with the anti-β mAb ARC5, indicating that pre-fractionation by differential centrifugation already provided an enrichment for plasma membranes (see below). After octylglucoside extraction, we found that β subunits could be immunoprecipitated exclusively from the low density, top region of the gradient (fractions 2, 3; Fig. 2A).

To further investigate the subcellular origin of those fractions associated with detergent insolubility of β subunits, homogenates were again subjected to differential centrifugation, followed by flotation gradient centrifugation. Fractions were taken from the top and divided into two aliquots, followed by sedimentation of the membranes. One half was processed as shown above (Fig. 2A), the second half was resuspended and incubated with WGA agarose beads (Fig. 3A). Vesicles derived from plasma membranes bind to wheat germ lectin via their exposed carbohydrates, whereas vesicles derived from TGN membranes and transport vesicles largely fractionate inside-out, preventing their binding to wheat germ lectin [14]. When we analyzed the WGA bead-bound material after solubilization in octylglucoside, we found that β subunits were recovered exclusively from fractions 2 and 3, corresponding to the occurrence of Triton-resistant β subunits in the same fractions. Immunoblotting analysis of pelleted membranes from gradient fractions demonstrated that the vast majority of α-$Na^+/K^+$-ATPase, a plasma membrane marker, is restricted to fractions 1–3 (Fig. 3B). We conclude that G protein β sub-

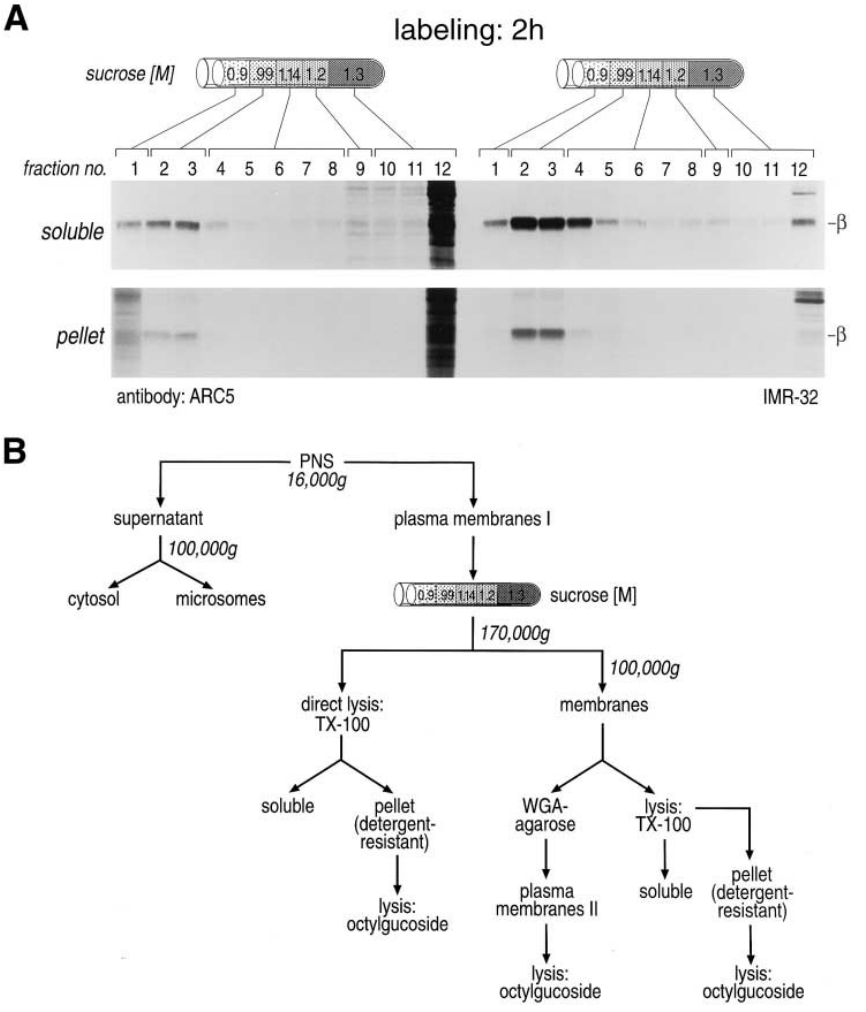


Fig. 2. Detergent-insoluble β subunits are restricted to low-density membrane fractions. A: IMR-32 cells were labeled for 2 h with 250 μCi [$^{35}$S]methionine, followed by homogenization as shown in Fig. 1. A postnuclear supernatant was loaded on a sucrose step gradient and resolved by equilibrium centrifugation. Fractions were taken from the top (see B for flow chart). One aliquot of each individual fraction was lysed immediately in TX-100-containing lysis buffer (left panel), followed by re-extraction of the pellet in octylglucoside. The other aliquot of each fraction was diluted first, and membranes were pelleted by centrifuging at 100 000×*g* for 1 h (right panel). Lysis in TX-100 and octylglucoside was the same as shown before (left panel). Immunoprecipitations were done in the presence of 0.2% SDS using mAb ARC5. Samples were analyzed by 12.5% SDS-PAGE. Note that fraction 12 (bottom) contains aggregates refractory to the homogenization conditions used. B: Flow chart for the purification of plasma membranes and detection of detergent-resistant membranes.

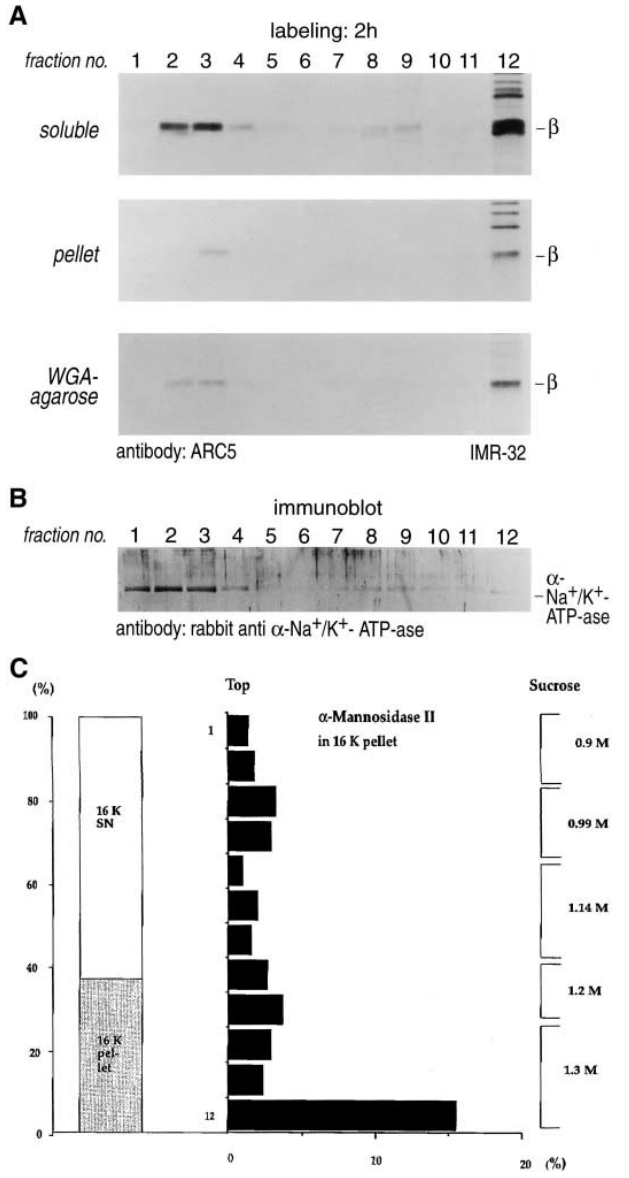


Fig. 3. Fractions containing detergent-insoluble β subunits exhibit the same low-buoyant density as membranes adsorbed to WGA beads. A: IMR-32 cells were labeled for 2 h with 250 μCi [$^{35}$S]methionine, and subjected to the same homogenization and flotation gradient centrifugation as shown in Fig. 2 (right panel, see also Fig. 2B). One aliquot of each individual fraction was then processed exactly as described (Fig. 2, right panel), whereas the remaining aliquot of each fraction was incubated with WGA beads. Plasma membranes adsorbed to WGA beads were then lysed in octylglucoside and subjected to immunoprecipitation with mAb ARC5. Samples were analyzed by 12.5% SDS-PAGE (A,B). B: The plasma membrane marker α-Na$^+$/K$^+$-ATPase is detectable in fractions 1–3, codistributing with WGA-absorbed membranes and detergent-insoluble β subunits. Equal volumes of each individual fraction as obtained in A were subjected to electrophoresis and transferred to nitrocellulose. The filter was incubated with rabbit anti α-Na$^+$/K$^+$-ATPase antiserum. C: Minimal α-mannosidase II activity in fractions enriched for plasma membranes. After centrifugation of a PNS at 16000×$g$, the pellet accounts for 35% of the mannosidase activity in the PNS. After flotation gradient centrifugation of the 16000×$g$ pellet, each individual fraction 1–3 contains less than 3% of the total α-mannosidase II activity (PNS: 100%).

units found in the top fractions of the gradient after differential extraction in TX-100 and octylglucoside are associated with plasma membranes.

The occurrence of detergent-resistant membranes has also been reported for TGN, but likely involves mechanisms distinct from those that apply to plasma membranes [6]. The combination of immunoblotting and enzyme marker assays showed that the distributions of marker enzymes for plasma membranes and Golgi membranes are clearly distinct. The distribution of the Golgi marker enzyme α-mannosidase II revealed that 35% (PNS: 100%) of this activity remained in the 16000×$g$ pellet (Fig. 3C). After sucrose step gradient centrifugation, none of the plasma membrane containing fractions 1–3 contained more than 3% of the total mannosidase activity.

The location of G protein β subunits in continuously labeled cells does not necessarily reflect the processes and kinetics underlying the acquisition of detergent insolubility in living cells. We have been unable to observe detergent-insoluble β subunits other than those associated with plasma membranes (data not shown).

We conclude that cytosol-derived heterotrimeric G protein βγ subunits are inserted into detergent-resistant membranes shortly after synthesis directly at the plasma membrane.

The isoprenoid moiety of Gγ facilitates targeting to detergent-resistant membranes in yeast [15]. It is not known whether the isoprenoid anchor mediates such an interaction by means of a lipid-adaptor-protein association or if the βγ complex is inserted directly into the lipid bilayer. To further explore the mechanisms of βγ complex association with detergent-resistant membranes we made use of PMA, a potent activator of PKC, the application of which was shown to prevent the internalization of caveolae [8]. The effect of PMA on detergent-resistant membrane association of βγ complexes was tested in a pulse-chase experiment where we added PMA or its inactive stereoisomer PDD after 10 min of chase at 37°C (Fig. 4). We failed to observe any difference in the amount of detergent-resistant βγ complexes in cells treated with PMA compared to control cells.

The evidence provided in support of the presence of heptahelical receptors in caveolae is mostly based on morphological

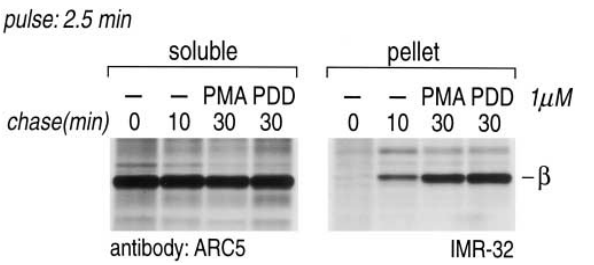


Fig. 4. The association of β subunits with detergent-resistant membranes is not affected by PMA-induced stimulation of PKC. IMR-32 cells were pulsed for 2.5 min with 500 μCi [$^{35}$S]methionine and chased for 10 min. At this chase point PMA or PDD (1 μM) was added and chase was continued for an additional 20 min. Cells were first lysed in TX-100-containing lysis buffer, followed by re-extraction of the pellet in octylglucoside. The amounts of radioactivity for individual chase points were normalized for the soluble and pellet fractions. Immunoprecipitations were done in the presence of 0.2% SDS using mAb ARC5, followed by analysis on a 12.5% SDS-PAGE.

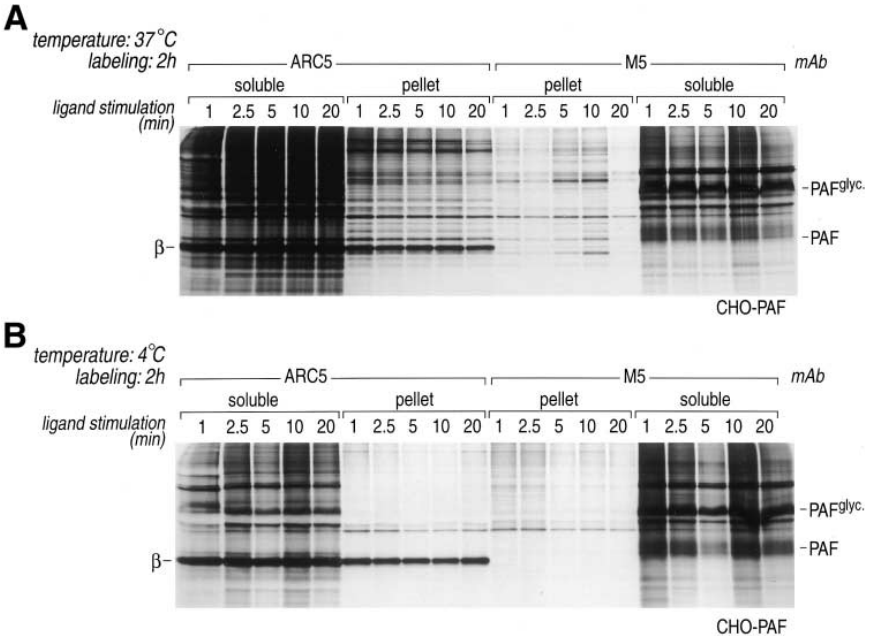


Fig. 5. The heptahelical receptor PAF fails to associate with detergent-resistant membranes. CHO cells transfected with the Flag-PAF receptor were labeled for 2 h with 250 μCi [$^{35}$S]methionine. Plates were washed and binding buffer was added containing 100 nM PAF. Incubations were performed at 4°C (B) or 37°C (A), followed by extraction as shown in Fig. 4. The amounts of radioactivity for individual ligand stimulation times were normalized for the soluble and the pellet fraction. G protein β subunits were recovered using the mAb ARC5, and PAF receptor was immunoprecipitated with the anti-Flag epitope mAb M5. Samples were analyzed on a 12.5% SDS-PAGE. Arrows indicate the positions of β subunits, PAF and glycosylated PAF receptor (PAF$^{glyc}$).

examinations [16,17]. We sought to explore this issue by examining the sequestration of the heptahelical PAF receptor after ligand stimulation. CHO cells stably transfected with an epitope-tagged PAF receptor were labeled for 2 h, and ligand was added at 100 nM for various times either at 4°C or at 37°C (Fig. 5). We failed to detect any PAF receptor in this domain under identical conditions where we find β subunits.

## 4. Discussion

In this report we describe the kinetics and intracellular pathways by which the βγ complexes of heterotrimeric G proteins are inserted into the plasma membrane and become detergent-resistant. Shortly after synthesis β subunits are recovered from the cytosol and from a light microsomal fraction; in both compartments β subunits are completely soluble (Fig. 1) upon extraction with non-ionic detergent buffer. The association with a plasma membrane-enriched fraction is detectable within 2.5–10 min, and we note that β subunits acquire detergent resistance in this domain with similar kinetics.

Further resolution of this plasma membrane-enriched $16000 \times g$ pellet by flotation on a sucrose step gradient was aimed at the separation of plasma membranes from Golgi membranes. In both domains the occurrence of detergent-resistant membranes has been reported [18,19]. After differential detergent extraction and β subunit-specific immunoprecipitation we find detergent-insoluble β subunits associated exclusively with plasma membrane of low buoyant density (Fig. 2A). In addition, we observe cofractionation of β subunits with WGA-absorbed material (Fig. 3A). We note that the remaining α-mannosidase II activity measured in the plasma membrane fractions accounts for less than 3% of the activity prior to fractionation (PNS: 100%; Fig. 3C). Therefore the β subunits recovered from these fractions are not derived from Golgi membranes.

The multistep procedure to isolate plasma membranes does not allow an exact quantitation of βγ complexes associated with detergent-resistant membranes. We conclude from our previous study that these βγ subunits are associated with α subunits that are susceptible towards pertussis toxin-mediated ADP ribosylation [11].

Our data suggest that cytosolically synthesized βγ complexes acquire detergent insolubility directly at the plasma membrane. Endosomal membranes can have low buoyant densities, comparable to those of plasma membranes. At present, we cannot rule out a rapid association of βγ complexes with an endosomal compartment. We consider this possibility unlikely, since detergent insolubility is caused largely by incorporation of glycosphingolipids, not known to be enriched in endosomal membranes. Detergent insolubility may be also caused by the association of βγ subunits with glycolipid rafts, which form as transport vesicles from the TGN and are targeted to the plasma membrane. In this view, the newly assembled cytosol-derived βγ subunits would use this pathway as a shuttle to the plasma membrane.

A similar pathway for trafficking of a cytosolic protein to plasma membranes has been reported for the Src-family kinase p59$^{fyn}$. Unlike the insertion of βγ complexes into plasma membranes coincident with acquisition of detergent resistance, a significant lag-phase was observed between plasma membrane binding and acquisition of detergent insolubility [20]. The differences in subunit modification, such as prenylation of the γ subunit versus myristoylation and palmitoylation of p59$^{fyn}$, may be related to the differences observed.

The rapid kinetics with which βγ complexes are inserted

into plasma membrane-associated detergent-resistant membranes implies that heptahelical receptors and cytosol-derived G protein subunits are targeted to their final destination differently. Although it has been established that heterotrimeric G proteins are retrieved from caveolae and detergent-resistant membranes, the occurrence of heptahelical receptors in such specialized domains remains controversial. Such association might depend on receptor species, cell line and ligand- or antibody-induced receptor triggering [9,21]. In our model system, the heptahelical PAF receptor did not partition into detergent-resistant membranes, whereas under the same conditions their signaling partners, the βγ complexes of G proteins, do so. We infer from this biochemical observation that, at least for some G protein signaling pathways, the functional relevance of G proteins in such domains awaits further validation.

The association of βγ complexes with DRM requires that the γ subunit receives an isoprenoid anchor which would facilitate the targeting of the βγ complex to such domains [22]. Since we observe a direct targeting of βγ complexes to the plasma membrane, this suggests that the requisite enzymatic activities for isoprenoid anchor maturation are located there.

The differences observed between the effects of PMA on caveolar architecture and function in a MA 104 kidney epithelial cell line and the lack of effect on βγ complex detergent insolubility raises several questions. First, it has been shown that prenylated model peptides fail to associate with microsomal membranes [23] after trypsin treatment of the membranes, indicating that this interaction is mediated by means of lipid-adaptor-protein interactions. To score for the possible influence of phosphorylation on such a proposed interaction, we made use of PMA to activate PKC, resulting in the phosphorylation of a multitude of substrates within the cell. This treatment does not alter the association of βγ complexes with detergent-resistant membranes, indicating that such a proposed adaptor function is unlikely to depend on phosphorylation status. PMA is also known to affect the biosynthesis and the hydrolysis of phospholipids. It was suggested that the profound effects of PMA on caveolar architecture were mediated via a lipid-metabolizing enzyme [8]. In conjunction with published results showing that neuroblastoma cells lack caveolae [24], the data reported in this study emphasize that caveolae and detergent-resistant membranes may exhibit not only morphological but also functional differences.

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