The Cytosolic Domains of Ca\(^{2+}\)-sensitive Adenylyl Cyclases Dictate Their Targeting to Plasma Membrane Lipid Rafts*

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Lipid rafts are specialized, cholesterol-rich domains of the plasma membrane that are enriched in certain signaling proteins, including Ca\(^{2+}\)-sensitive adenylyl cyclases. This restrictive localization plays a key role in the regulation of the Ca\(^{2+}\)-stimulable AC8 and the Ca\(^{2+}\)-inhibitable AC6 by capacitative calcium entry. Interestingly, AC7, a Ca\(^{2+}\)-insensitive AC, is found in the plasma membrane but is excluded from lipid rafts (Smith, K. E., Gu, C., Fagan, K. A., Hu, B., and Cooper, D. M. F. (2002) J. Biol. Chem. 277, 6025–6031). The mechanisms governing the specific membrane targeting of adenylyl cyclase isoforms remain unknown. To address this issue, a series of chimeras were produced between the raft-targeted AC5 and the non-raft-targeted AC7, involving switching of their major domains. The AC5-AC7 chimeras were expressed in HEK 293 cells and lipid rafts were isolated from the bulk plasma membrane by either detergent-based or non-detergent-based fractionation methods. Additionally, confocal imaging was used to investigate the precise cellular targeting of the chimeras. Surprisingly, the two tandem six-transmembrane domains of AC5 were not required for localization to lipid rafts. Rather, AC5 localization depended on the complete cytoplasmic loops (C1 and C2); constructs with mixed domains were either retained in the endoplasmic reticulum or degraded. Similar conclusions are drawn for the lipid raft localization of the Ca\(^{2+}\)/calmodulin-stimulable AC8; again, the C1 and C2 domains are critical. Thus, protein-protein interactions may be more important than protein-lipid interactions in targeting these calcium-sensitive enzymes to lipid rafts.

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The fluid mosaic model for the plasma membrane (PM)\(^1\) proposed a homogeneous lipid bilayer with which membrane proteins associated to varying degrees, depending on their overall lipid solubility. It is now becoming apparent that cell surface membranes are heterogeneous, containing “microdomains” of different lipid and protein composition, which are typified by lipid rafts (1). Lipid rafts are rich in cholesterol and sphingolipids, and they display a high concentration of signaling proteins, such as receptors, G-proteins, and effectors, including ion channels and adenylyl cyclases (ACs) (2, 3). However, by no means are all membrane-associated signaling proteins located in lipid rafts (4, 5). Understanding how this differential PM localization occurs may illuminate the significance of this targeting.

ACs are a class of integral membrane proteins that display heterogeneity in PM localization. There are nine membrane-associated AC isoforms that are regulated by a variety of factors, including phosphorylation, intracellular Ca\(^{2+}\), and GTP-regulatory proteins. AC1 and AC8 are activated by Ca\(^{2+}\) in a calmodulin-dependent manner, whereas AC5 and AC6 are inhibited by Ca\(^{2+}\), independently of calmodulin (6). Accumulating evidence from heterologous expression studies is beginning to suggest that Ca\(^{2+}\)-sensitive ACs (specifically AC5, AC6, and AC8) are enriched in lipid rafts, whereas Ca\(^{2+}\)-insensitive ACs (AC2, AC4, and AC7) (4, 7, 8) are excluded from rafts and reside in the bulk PM. This differential localization may be directly related to specific properties of AC regulation, since the residence of Ca\(^{2+}\)-regulatable ACs in lipid rafts is crucial for their responsiveness to physiological elevations in cytosolic Ca\(^{2+}\).

Specifically, the disruption of lipid rafts by depletion of cholesterol abolishes the regulation of AC6 and AC8 by capacitative calcium entry (CCE) (7, 9). The significance of the presence of protein kinase C-stimulated but Ca\(^{2+}\)-insensitive ACs (AC2, AC4, and AC7) in nonraft domains has not been addressed. However, it seems reasonable to speculate that differential PM localization may contribute to type-specific regulation of AC.

The targeting of AC8 to the PM requires interactions between its two transmembrane domains (TMDs) (10). Thus, constructs encoding separate transmembrane cassettes are retained in the endoplasmic reticulum (ER) when expressed separately, but they are delivered to the PM when expressed together. However, nothing is known of the molecular determinants that target ACs to microdomains of the PM. In the present study, we sought to identify the structural elements of AC5 that determine its localization to lipid rafts. To address this issue, we generated a number of chimeric constructs between the raft-targeted AC5, and AC7, which is excluded from rafts, and examined the disposition of the chimeric forms when heterologously expressed in HEK 293 cells. A nondetergent, sonication-based method for raft enrichment, combined with confocal microscopic analysis, was used to confirm the cellular localization of the chimeras. The presence of AC5-AC7 chimeras suggested to be in lipid rafts by the nondetergent method was also assessed by a raft preparation method involving cold extraction with the detergent, Triton X-100. Surprisingly, for an integral membrane protein, the targeting of AC5 to lipid rafts is determined by the C1 and C2 domains.
rafts, generated by either detergent or nondetergent extraction, depended only on the cytoplasmic domains (C1 and C2) and was independent of the TMDs. The importance of the cytosolic domains was confirmed with AC8, which was also targeted to rafts. Combinations of half-molecules of AC8 lacking the cytosolic domains were enriched in nonraft domains of the PM, whereas combinations of half-molecules that included the cytosolic domains were delivered to rafts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Purified cationic antibody and monoclonal flotillin antibody were obtained from BD Transduction Laboratories (Erembodegem, Belgium). Polyclonal AC7 antibody and c-Myc monoclonal antibody (clone 9E10) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Penta-His monoclonal antibody and Effectene were from Qiagen (Crawley, UK). Green fluorescent protein monoclonal antibody was from Zymed Laboratories Inc. (San Francisco, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Amersham Biosciences, and horseradish peroxidase-conjugated goat anti-mouse IgG was from Promega (Madison, WI). Paraformaldehyde was obtained from TAAB Laboratories (Aldermaston, UK). Alexa Fluor 546 goat anti-mouse IgG and Alexa Fluor 488 donkey anti-goat IgG were from Molecular Probes (Leiden, The Netherlands). Tissue culture medium was purchased from Sigma.

cDNA Plasmid Constructs—The cDNA clones of rabbit AC5 (GenBank accession number Z39371) and bovine AC7 (GenBank accession number Z49806) were used to construct all chimeric constructs. The constructs AC7(AC5 Nt, C1, C2) and AC7(AC5 C1, C2) were as described earlier (11). In AC5ΔC2b, the seven C-terminal amino acids, NGGPPSL, were removed, and in AC5(AC5 C2b) this stretch was replaced by the 13 C-terminal amino acids from AC7 (CTDFTAKFGQGLGLN). The reverse was generated with AC7; i.e., in AC7ΔC2b, CTDTAKFGQGLGLN was replaced, and in AC7(AC5 C2b) this stretch was replaced by NGGPPSL, AC7(AC5 C1a, C2) had the composition AC7–1 to 201-VD–AC5–406 to 645-TR–AC7–473 to 832-LE–AC5–1013 to 1264.

Caveolin and noncaveolar membranes were isolated using a method described above. HEK 293 cells, which stably expressed the AC constructs, were also plated onto poly-L-lysine-precoated coverslips and grown for an identical period of time (20 h). Cells were pelleted (195 g) 1 day before transfection. In addition, stably transfected HEK 293 cells were visualized on a Zeiss Axiovert LSM510 confocal microscope, by maintaining the cells in the presence of 400 ng/ml geneticin sulfate (G418).

**Cell Culture and Transfection**—HEK 293 cells (European Collection of Cell Cultures, Porton Down, UK) were grown in minimum essential medium supplemented with 10% fetal bovine serum and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Cells were plated on 100-mm dishes at ~70% confluence 1 day before transfection with 2 μg of cDNA using Effectene according to the manufacturer’s procedures. Transiently transfected cells were used 2 days post-transfection. In addition, stably transfected HEK 293 cells were established by maintaining the cells in the presence of a 400 μg/ml genetin sulfate (G418).

**Nondetergent Isolation of Caveolar and Noncaveolar Membranes**—Caveolar and noncaveolar membranes were isolated using a method that has been described previously (12). Briefly, transfected HEK 293 cells were pelleted (195 × 5, 5 min) and resuspended in ice-cold sodium carbonate solution (500 mM Na2CO3, 150 mM NaCl, pH 11.0) before sonication (Sonics Diamembratter; Fisher) at 4 °C, 30 s at setting 2, 30 s at setting 3, and 30 s at setting 4 with 3 min between each sonication (13). The suspension was transferred to a tightly fitting Dounce homogenizer and homogenized with 20 strokes. The homogenate was adjusted to 40% sucrose by the addition of 60% MES buffer and centrifuged in a Beckman SW55 rotor at 24,000 rpm for 16 h at 4 °C. Fractions (10 × 0.5 ml) were collected from the top of the gradient. The sucrose concentration of an aliquot (40 μl) of each fraction was measured in an Abbe refractometer. The remainder of the isolated fractions were diluted in 5 volumes of MES buffer and centrifuged in a Beckman SW55 rotor at 50,000 rpm for 1 h at 4 °C. The pelleted membranes were resuspended in 1% SDS containing 8 μM urea, from which 30 μl was removed for protein determination (bicinchoninic acid assay kit; Sigma). Finally, samples were suspended in loading buffer (final concentration: 62.5 mM Tris, 150 mM dithiothreitol, 20% glycerol, final Trx-X–100 concentration of 0.1%. The sample was homogenized with 20 strokes of a tightly fitting Dounce homogenizer and left on ice for 30 min, followed by adjustment to 40% sucrose with the addition of 1 ml of 60% sucrose TNE. The rest of the procedure was essentially identical to that described for nondetergent isolation of membranes, except that TNE was the buffer used throughout the procedure. Pelleted samples were resuspended in 1% SDS and suspended in gel loading buffer (as described above) but without urea.

**Immunoblotting Assays**—Proteins were resolved using SDS-PAGE 7.5 and 12% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. (All AC5-AC7 gels were prepared with 8 μM urea, since this reduced immunoreactive smearing and reduced adenylyl cyclase aggregation). The polyvinylidene difluoride membrane was incubated in blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl (TBS)) containing 5% skimmed milk powder for 30 min, followed by two 10-min washes in TBS supplemented with 0.05% (v/v) Tween 20 (TBSB). Membranes were incubated overnight at room temperature with anti-caveolin polyclonal antibody (1:5000), anti-flotillin mAb (1:5000), anti-Ga, of polyclonal antibody (1:1000), anti-c-Myc mAb (1:1000), anti-GFP mAb (1:1000), or anti-penta-His mAb (1:2000) in TBS containing 1% skimmed milk powder (or blocking buffer). The membrane was incubated (2 × 10 min) in TBSB and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 dilution of stock) or goat anti-mouse IgG conjugated to horseradish peroxidase (1:3000) in antibody buffer for 1 h. Finally, the membranes were washed in TBSB (2 × 10 min), rinsed in TBS, and treated with ECL reagent (Amersham Biosciences) (in detergent experiments, ECL plus was used) and exposed to Kodak X-Omat Blue film.

**Immunofluorescence**—HEK 293 cells (1 × 106 cells) were plated onto poly-l-lysine-coated glass coverslips (22-mm diameter; BDH) and cultured for 24 h. Cells were then transfected with 0.4 μg of DNA using the Effectene method described above. HEK 293 cells, which stably expressed the AC constructs, were also plated onto poly-l-lysine-precoated coverslips and grown for an identical period of time (i.e. 72 h). Cells were then washed with phosphate-buffered saline (PBS; 12.1 mM Na2HPO4, 4 mM KH2PO4, and 130 mM NaCl, pH 7.4) and fixed using 4% paraformaldehyde (1 h, 20 °C). After fixing, cells were washed further in PBS and permeabilized (1 h, 20 °C) with PBS containing 0.2% Triton X-100 and 1% goat serum (blocking solution) and incubated (12 h, 20 °C) with anti-c-Myc mAb (1:300 dilution) or anti-AC7 polyclonal antibody (1:1000 dilution). Cells were then washed with phosphate-buffered saline (PBS; 12.1 mM Na2HPO4, 4 mM KH2PO4, and 130 mM NaCl, pH 7.4) and fixed using 4% paraformaldehyde (1 h, 20 °C). After fixing, cells were washed further in PBS and permeabilized (1 h, 20 °C) with PBS containing 0.2% Triton X-100 and 1% goat serum (blocking solution) and incubated (12 h, 20 °C) with anti-c-Myc mAb (1:300 dilution) or anti-AC7 polyclonal antibody (1:1000 dilution).
Preparation of Crude Membranes—HEK 293 cells expressing the various AC constructs that were grown until 70% confluent in 100-mm diameter Petri dishes were detached with PBS containing 0.03% EDTA and centrifuged (195 × g, 5 min). The supernatant was removed, and the pellet was resuspended in 2 ml of HB buffer (2 mM MgCl₂, 1 mM EDTA, 50 mM Tris, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 mM benzamidine, 1 μg of DNase, pH 7.4) and lysed by pressing the cell suspension through a 0.22-gauge needle 10 times. After further centrifugation (195 × g, 5 min) and dissociation, the lysate was centrifuged (17,257 × g, 15 min, 4 °C). The supernatant was removed, and the pellet was resuspended in 25–500 μl of assay buffer (40 mM Tris-Cl, 800 μM EGTA, 0.25% bovine serum albumin (fraction V), pH 7.4) and stored in liquid nitrogen until required.

Measurement of Adenylyl Cyclase Activity—AC activity was measured as described previously (17). Briefly, HEK 293 membranes (see “Preparation of Crude Membranes”) were incubated (20 min, 30 °C) in the presence of 12 mM phosphocreatine, 1.4 mM MgCl₂, 40 μM GTP, 100 μM camp, 100 μM ATP, 25 units/ml creatine kinase, 70 mM Tris-Cl, 500 μM isobutylmethylxanthine, 1 μCi of [α-32P]ATP, 10 μM forskolin, and Ca²⁺ (see “Determination of Free Ca²⁺ Concentrations”). Reactions were terminated, and the [32P]-cAMP formed was quantified as described previously using [H]cAMP as a recovery marker (7, 18).

Determination of Free Ca²⁺ Concentrations—Free Ca²⁺ concentrations were determined using an optimized version of a described previously nondetergent method (17). Briefly, HEK 293 membranes (see “Experimental Procedures”) were incubated with 12 mM phosphocreatine, 1.4 mM MgCl₂, 40 μM GTP, 100 μM camp, 100 μM ATP, 25 units/ml creatine kinase, 70 mM Tris-Cl, 500 μM isobutylmethylxanthine, 1 μCi of [α-32P]ATP, 10 μM forskolin, and Ca²⁺ (see “Determination of Free Ca²⁺ Concentrations”). Reactions were terminated, and the [32P]-cAMP formed was quantified as described previously using [H]cAMP as a recovery marker (7, 18).

RESULTS

Localization of AC5 in Lipid Rafts and of AC7 in Nonraft, Bulk PM—When heterologously expressed in HEK 293 cells, Ca²⁺-regulatable AC8 and AC5 occur exclusively in lipid rafts, whereas the Ca²⁺-insensitive AC7 is found only in the nonraft, bulk membrane (7). As a prelude to investigating the mechanisms underlying differential PM targeting of AC isoforms, HEK 293 cells were transfected with Ca²⁺-inhibitable AC5 (Myc-tagged) and Ca²⁺-insensitive AC7 (His-tagged), and their localization was examined. Transfected cells were fractionated using an optimized version of a described previously nondetergent-based method (i.e. sonication in sodium carbonate (see “Experimental Procedures”)) and sucrose gradient fractionation (12). Ten equivalent volume fractions were collected from the sucrose equilibrium density gradient, and sucrose and protein concentrations were determined for each fraction (Fig. 1A). Compared with nonraft bulk membranes, lipid rafts have a higher lipid/protein ratio, permitting their isolation because of their increased buoyancy in sucrose density gradients (21). Caveolin, flotillin, and Gαi are specific raft marker proteins (22–24), and their immunoreactivity was most pronounced in fraction 4, corresponding to ~20% sucrose (Fig. 1, A and B). HEK 293 cells expressing Myc-tagged AC5 were fractionated, and Myc immunoreactivity was clearly enriched in the fraction that displayed the greatest immunoreactivity of raft marker proteins (Fig. 1B). Lipid raft microdomains also occur in Golgi membranes (25), and immunofluorescent confocal analysis confirmed that AC5 was localized predominantly at the PM and not in internal membranes in the majority of cells (Fig. 1B).

The nonraft protein β-adaptin, a component of clathrin-coated pits, is excluded from buoyant membranes and occurs only in the bulk membrane. β-adaptin immunoreactivity demonstrated that the nonraft, bulk PM corresponded to fractions 7 and 8 (Fig. 1C). These fractions sediment at ~35–40% sucrose, and they account for the majority of the protein in the equilibrium density gradient (Fig. 1A). His-tagged AC7 was excluded from the lipid raft fraction and was detected only where β-adaptin immunoreactivity occurred (Fig. 1C). Endosomal reticulum membranes also sediment at ~35–40% sucrose; confocal analysis was therefore used to confirm that AC7 predominantly resided in the PM, with only weak intracellular labeling (Fig. 1C).

Because AC5 and AC7 occurred in separate, identifiable domains of the PM, the region(s) involved in targeting AC to lipid rafts in HEK 293 cells could be explored by generating a series of chimeric constructs containing domains from AC7 inserted into AC5, Myc-tagged at the C terminus.

The Transmembrane Domains and N Terminus Are Not Involved in AC5 Targeting to Lipid Rafts—The targeting of the platelet-derived growth factor receptor to lipid rafts has been described to be through the association of its transmembrane domains (TMDs) with cholesterol (27). Additionally, the localization of influenza hemagglutinin to rafts is abolished upon mutation of amino acids within the exoplasmic domain (28). The two TMDs of ACs are poorly conserved between individual AC species, and it therefore seemed reasonable to consider that the TMDs might be important in membrane targeting. The TMDs of AC5 were exchanged with the corresponding regions from AC7. The resulting chimera (AC7[AC5 Nt, C1, C2]) (Fig. 2A) was expressed in HEK 293 cells, and its membrane localization was examined by the nondetergent method for raft enrichment. Surprisingly, AC7[AC5 Nt, C1, C2] with an approximate molecular Mₚ of 130 kDa, was clearly enriched in the buoyant membrane fraction (Fig. 2A). Additionally, confocal imaging demonstrated that AC7[AC5 Nt, C1, C2] was largely restricted to the PM (Fig. 2A). This result suggests that the cytoplasmic domains and not the TMDs of AC5 are important in raft localization.

To explore the cytoplasmic regions of AC5 with a possible role in targeting to rafts, a chimeric construct in which only the N terminus and TMDs of AC5 were replaced with comparable domains from AC7 (AC7[AC5 C1, C2]) was expressed in HEK 293 cells (Fig. 2B). Fractionation demonstrated that AC7[AC5 C1, C2] was restricted to the buoyant membrane (Fig. 2B), and confocal analysis confirmed that targeting was predominantly localized to the PM, with only weak intracellular labeling (Fig. 2B). These results suggest that the N terminus and TMDs are not required for raft localization and that the cytoplasmic domains (C1 and C2) are the likely mediators in raft targeting of AC5.

The C1b Domain of AC5 Is Required for Correct Protein Handling—The catalytic domains (C1a and C2a) are well conserved between different AC isoforms; however, the C1b domain is much less conserved. Consequently, an AC5-AC7 chimera was constructed in which the N terminus and TMDs from AC7 were replaced with comparable domains from AC5 (AC7[AC5 Nt, C1, C2]) (Fig. 3). Fractionation revealed that AC5[AC7 C1, C2] was retained within the ER membrane, and AC7[AC5 Nt, C1, C2] with an approximate molecular Mₚ of 130 kDa, was detectable in fraction 4. The reason for the subtle difference in Mₚ between fractions 4 and 7 is not clear, but it most likely arises from differences in post-translational modification (30). Confocal analysis revealed that AC7[AC5 C1a, C2] was retained within the ER membrane, and no PM or Golgi labeling was seen (Fig. 3). This result implies that the C1b domain of AC5 is required for correct protein handling and targeting to the PM.

Role of the C2b Domain in Processing of AC5 and AC7—The role of the C2b domain in targeting was also investigated. This domain is also poorly conserved; it corresponds to 7 amino acids in AC5 and 13 amino acids in AC7. Initially, an AC5 mutant in which the C2b domain was deleted (AC5ΔC2b) was explored (Fig. 4A). Fractionation revealed that AC5ΔC2b occurred only in the pellet and at a lower than the predicted molecular weight
There was no detectable immunofluorescence on confocal examination (data not shown).

Given the dramatic effect of C2b deletion on membrane association and survival of full-length AC5, we wondered whether the addition of the C2b domain from AC7 to AC5 (AC5[AC7 C2b]) would rescue membrane targeting and yield a protein of the correct Mr. Indeed, a protein of the anticipated Mr was produced, which fractionated with nonraft bulk membrane. However, confocal imaging revealed that AC5[AC7 C2b] was not targeted to the PM and was predominantly expressed in the ER, with no observable increase in membrane fluorescence at points of cellular contact (Fig. 4B).

To determine whether we could generalize the role of the C2b domain from AC5 to AC7, an AC7 construct lacking a C2b domain (AC7ΔC2b) (Fig. 4C) was expressed in HEK 293 cells, and its localization was examined. AC7ΔC2b was restricted to the nonraft bulk membrane fraction with the expected Mr, and, by confocal analysis, it was localized at the PM, with a visible increase in membrane fluorescence at points of cellular contact. This construct therefore had an identical expression profile to that of wild-type AC7, which showed that, unlike AC5, an intact C2b domain was not essential for membrane insertion or correct protein handling (Fig. 4C).

Replacing the C2b Domain of AC7 with That from AC5 Confers Both Raft and Nonraft Bulk Membrane Localization of AC7—The results above clearly suggest that the C2b domains

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**Fig. 1. Localization of AC5 to lipid rafts and AC7 to bulk plasma membrane.** Transfected HEK 293 cells were lysed and prepared for sucrose equilibrium density gradient centrifugation as described under “Experimental Procedures.” A, the percentage of protein content (as compared with total recovered protein) and percentage of sucrose from 10 0.5-ml fractions (n = 4 ± S.E.) are indicated. B, fractions (lanes 3–8) from HEK 293 cells transfected with Myc-tagged AC5 were resolved by SDS-PAGE and immunoblotted with antibodies raised against caveolin (cav), Gaα-solf, flotillin, and Myc (α-myc). The same cells were fixed and processed for immunofluorescent confocal imaging with an antibody raised against Myc. C, fractions (lanes 3–8) from HEK 293 cells transfected with His-tagged AC7 were resolved by SDS-PAGE and immunoblotted with antibodies raised against β-adaptin and His (α-his). The same cells were fixed and processed for immunofluorescent confocal imaging with an antibody raised against AC7.

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(Fig. 4A). There was no detectable immunofluorescence on confocal examination (data not shown).

Given the dramatic effect of C2b deletion on membrane association and survival of full-length AC5, we wondered whether the addition of the C2b domain from AC7 to AC5 (AC5[AC7 C2b]) (Fig. 4B) would rescue membrane targeting and yield a protein of the correct Mr. Indeed, a protein of the anticipated Mr was produced, which fractionated with nonraft bulk membrane. However, confocal imaging revealed that AC5[AC7 C2b] was not targeted to the PM and was predominantly expressed in the ER, with no observable increase in membrane fluorescence at points of cellular contact (Fig. 4B).

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of AC5 and AC7 do not fulfill identical roles in terms of protein handling and membrane targeting. However, although the C2b domain of AC5 seems essential for correct ER handling, whether this domain is involved in raft localization cannot be assessed from the previous experiment. Since targeting of AC7 was independent of its C2b domain, we exchanged the C2b domain of AC7 for that of AC5 (AC7[AC5 C2b]) to determine whether this would target AC7 to rafts. This construct displayed an interesting fractionation profile, still predominantly restricted to the bulk membrane but with detectable immuno-reactivity in the buoyant fraction 4. Three distinct bands with approximate Mr of 100, 110, and 230 kDa occurred in fractions 6 and 7 (Fig. 5A). The two lower molecular weight bands probably arise from differences in post-translational modification (29); the 230-kDa band may be a dimeric form (30). Interestingly, in the buoyant membrane fraction, an immuno-reactive band of ~110 kDa was observed, which may represent a glycosylated form. This is similar to that observed with AC7[AC5 C1a, C2] (Fig. 3) and is suggestive that a form of post-translational modification occurs in the transition from heavier membrane fractions to cholesterol-rich membranes. Confocal analysis revealed both PM and intracellular localization, suggesting that AC7[AC5 C2b] is partially targeted to the PM but is not restricted to the bulk membrane component, since immunoreactivity is also detected in lipid rafts (Fig. 5A).

**Fig. 2.** A role for the cytosolic domains, but not the N terminus or transmembrane domains, in AC5 localization to rafts. HEK 293 cells transfected with the indicated Myc-tagged AC5-AC7 chimeras were lysed and subjected to sucrose equilibrium density gradient centrifugation as described under “Experimental Procedures.” Fractions (lanes 3–8) were resolved by SDS-PAGE and immunoblotted with antibodies raised against caveolin (cav) and Myc (α-myc). The same cells were fixed and processed for immunofluorescent confocal imaging with an antibody raised against Myc. A, the TMDs of AC5 exchanged with the TMDs from AC7 (AC7[AC5 Nt, C1, C2]). B, the N terminus and TMDs of AC5 exchanged with the corresponding domains from AC7 (AC7[AC5 C1, C2]).

**Fig. 3.** The native C1b domain of AC5 is required for correct protein handling. HEK 293 cells transfected with a Myc-tagged AC5-AC7 construct consisting of the N terminus, TMDs and C1b domain derived from AC7, and the C1a and C2 domains derived from AC5 (AC7[AC5 C1a, C2]) were lysed and prepared for sucrose equilibrium density gradient centrifugation as described under “Experimental Procedures.” Fractions (lanes 3–8) were resolved by SDS-PAGE and immunoblotted with antibodies raised against caveolin (cav) and Myc (α-myc). These cells were imaged by immunofluorescent confocal microscopy with an antibody raised against Myc.
nonconserved C1b and C2b domains from AC5 would be delivered to rafts. Fractionation and confocal analysis revealed that AC7[AC5 C1b, C2b] was only present as a degraded product (Fig. 5B). The chimeric construct produced only weak immunoreactivity in fraction 8. Additionally, there was no observable immunofluorescence on confocal examination (data not shown). Therefore, it seems clear that, to permit raft localization of AC5, full-length native C1 and C2 domains are necessary.

**Plasma Membrane-targeted AC5, AC7[AC5 Nt, C1, C2], and AC7[AC5 C1, C2] Reside in Detergent-resistant Microdomains**—To confirm that the conclusions reached so far did not depend on the method used for raft isolation, another method
was employed (i.e., insolubility in cold Triton X-100). Indeed, the traditional definition of a lipid raft related to its insolubility in 1% Triton X-100 and flotation at 20% on a sucrose gradient (31).

Purified PMs that floated at 30–37% on a continuous sucrose gradient were collected and incubated in cold TNE containing Triton X-100 and subjected to sucrose equilibrium density centrifugation. When detergent was omitted from the procedure, caveolin immunoreactivity was only detectable in fraction 7, which corresponded to the bulk PM fraction, and no raft marker proteins were seen at 20% sucrose (fraction 4; Fig. 6A).

When Triton X-100 was included at a 10:1 (w/w) detergent/protein ratio, caveolin immunoreactivity was highly enriched in detergent-resistant membranes, corresponding to lipid rafts (fraction 4) (Fig. 6B–D). In this detergent-resistant fraction, AC5, AC7[AC5 Nt, C1, C2], and AC7[AC5 C1, C2] were all enriched (Fig. 6B–D). When the detergent/protein ratio was

**FIG. 6.** The N terminus and TMDs of AC5 are not required for localization to detergent resistant microdomains. Purified plasma membranes from HEK 293 cells transfected with the indicated Myc-tagged AC5-AC7 chimeras were incubated in the absence (A) or presence (B–D) of cold Triton X-100 and prepared for sucrose equilibrium density gradient centrifugation as described under “Experimental Procedures.” A, fractions (lanes 3–10) were resolved by SDS-PAGE and immunoblotted with antibodies raised against caveolin (cav). B–D, fractions (lanes 3–10) were resolved by SDS-PAGE and immunoblotted with antibodies raised against caveolin (cav) and Myc (α-myc). B, full-length AC5. C, the TMDs of AC5 were exchanged with the TMDs from AC7 (AC7[AC5 Nt, C1, C2]). D, the N terminus and TMDs of AC5 were exchanged with the corresponding domains from AC7 (AC7[AC5 C1, C2]).
increased, lipid rafts no longer resisted cold extraction and were solubilized; caveolin and AC5 were then detected only in fractions 7–10 (data not shown). Thus, AC5, AC7[AC5 Nt, C1, C2], and AC7[AC5 C1, C2] are enriched in lipid rafts prepared either by sonication or cold detergent extraction.

**Plasma Membrane-targeted AC5, AC7[AC5 Nt, C1, C2], and AC7[AC5 C1, C2] Are Functional, Ca\(^{2+}\)-inhibitable Enzymes**—Of all of the AC5-AC7 chimeras examined, only AC7[AC5 Nt, C1, C2] and AC7[AC5 C1, C2] were clearly enriched in PM lipid rafts. These chimeras contained the C1a and C2a domains of AC5, which is the site of high affinity Ca\(^{2+}\) inhibition (32). To demonstrate that these constructs were functional enzymes, with the properties expected of constructs bearing the catalytic domains of AC5, AC activity was measured at a range of Ca\(^{2+}\) concentrations in crude membranes prepared from HEK 293 cells expressing AC5, AC7[AC5 Nt, C1, C2], or AC7[AC5 C1, C2] as well as AC7. AC5 and AC6 are the “Ca\(^{2+}\)-inhibitable ACs,” displaying inhibition by submicromolar \([\text{Ca}^{2+}]\) (33). Half-maximal inhibition of AC5 occurs at 0.3 μM free Ca\(^{2+}\) (Fig. 7), a value similar to that observed previously with AC6 (34). Both AC7[AC5 Nt, C1, C2] and AC7[AC5 C1, C2] displayed an almost identical profile for inhibition by low concentrations of Ca\(^{2+}\) as the wild type AC5 (half-maximal inhibition at 0.35 and 0.28 μM, respectively; Fig. 7). AC7, along with AC2 and AC4, is a “Ca\(^{2+}\)-insensitive” AC and is not inhibited by submicromolar \([\text{Ca}^{2+}]\) (33, 34). Substantial inhibition of AC7 by Ca\(^{2+}\) is only seen at submillimolar free \([\text{Ca}^{2+}]\) (half-maximal Ca\(^{2+}\) inhibition is estimated at 300 μM free Ca\(^{2+}\); Fig. 7, inset). All membrane-bound ACs display inhibition *in vitro* at this high level of free Ca\(^{2+}\), which is not considered to be of physiological relevance (32, 34). Although AC7[AC5 C2b] was partly represented in lipid rafts (Fig. 5a), it contains the catalytic domain of AC7, which cannot be inhibited by submicromolar \([\text{Ca}^{2+}]\) (see Fig. 7, inset). The present functional results, then, are as anticipated, unless the construction of the chimeras had induced some distortion of the catalytic domain.

**The Transmembrane Domains Are Not Responsible for Plasma Membrane Lipid Raft Localization of Ca\(^{2+}\)/Calmodulin-stimulable AC8**—The results so far have shown that the cytoplasmic domains, C1 and C2, of AC5, and not the N terminus or TMDs, govern lipid raft localization. To determine whether this characteristic applied to other raft-localized ACs, we examined the major domains responsible for AC8 localization to lipid rafts. When heterologously expressed in HEK 293 cells, AC8 resides in lipid rafts, a property that is essential for its responsiveness to CCE (7). As with AC5, deletion of the N terminus of AC8 does not alter its raft localization (7), which implies that raft targeting is mediated through the TMDs or cytoplasmic C1 and C2 domains. Earlier studies had shown that both transmembrane cassettes of AC8, when expressed separately without the cytosolic C1 or C2 domains, are retained in the ER (10). However, co-expression of the two AC8 transmembrane cassettes results in the formation of a tight complex between the TMDs and their delivery to the PM, as shown by co-immunoprecipitation and fluorescence resonance energy transfer analysis of GFP-tagged constructs (10). We sought to extend these observations by identifying the PM location of co-expressed transmembrane cassettes of AC8. HEK 293 cells, expressing, alone or in combination, the N terminus and first transmembrane cassette of AC8 (8NTm1/GFP) and the second transmembrane cassette of AC8 (GFP/8Tm2) were fractionated by the detergent-free method. Expressed alone, 8NTm1/GFP was present primarily in nonraft intracellular membranes and displayed the anticipated M<sub>r</sub> of 65 kDa (Fig. 8A). GFP/8Tm2 was also intracellular but was associated with buoyant membrane fractions and displayed the anticipated M<sub>r</sub> of 55 kDa (Fig. 8B). However, confocal imaging demonstrated that GFP/8Tm2 was not associated with the PM and was intracellular in localization. When expressed together, the two transmembrane cassettes yielded predominantly fluorescence at the PM, with only a small signal arising from intracellular sources (Fig. 8C) as reported earlier (10). The fractionation profile of the co-expressed transmembrane cassettes was very informative. The 65-kDa band (corresponding to 8NTm1/GFP) was seen in all fractions but was most prominent in fraction 7, which corresponded to the bulk membrane fraction (Fig. 8C); the 55-kDa band (corresponding to GFP/8Tm2) also displayed the greatest immunoreactivity in this fraction (Fig. 8C). The higher M<sub>r</sub> bands observed are most likely due to dimerization of the TMDs.3 8NTm1/GFP and GFP/8Tm2 are found in close association at the PM when co-expressed (10). Although GFP/8Tm2 was enriched in intracellular lipid rafts, implying that 8Tm2 may in part be responsible for raft localization of AC8 (Fig. 8B), this was clearly not sufficient to target the two interacting TMDs to PM lipid rafts (Fig. 8C), suggesting that additional factors are necessary.

**The Cytoplasmic C1 and C2 Domains of AC8 Are Required for Plasma Membrane Lipid Raft Localization**—To address the potential role of the C1 and C2 domains of AC8 in lipid raft localization, the first half of AC8, consisting of the N terminus, the first transmembrane cassette, and the C1 domain (8NTm1C1/GFP), was expressed alone or in combination with the second transmembrane cassette of AC8 with its C2 domain (GFP/8Tm2C2). Expressed separately, 8NTm1C1/GFP displayed only an intracellular localization (Fig. 9A). This construct (~100 kDa) was not seen in the buoyant membrane fraction but was associated with the bulk membrane and particulate fractions (Fig. 9A). GFP/8Tm2C2 showed an intracellular localization, whereas fractionation revealed that this construct (~85 kDa) was detectable in rafts, bulk membrane, and particulate fractions (Fig. 9B). Very interestingly, when the two halves of AC8 were co-expressed, 8NTm1C1/GFP immunoreactivity (~100 kDa) was only found with the buoyant membrane fraction, which implied that the presence of GFP/8Tm2C2 was sufficient to redistribute 8NTm1C1/GFP from the bulk membrane to lipid rafts (Fig. 9C). Confocal examination revealed that certain cells displayed restrictive PM labeling, whereas other cells had both PM and intracellular labeling. This is consistent with the fractionation profile of GFP/8Tm2C2 (~85 kDa), which was present in all fractions (Fig. 9C). This finding reflects the fact that Tm2 homodimerizes and remains intracellular, whereas Tm1 does not homodimerize (10). Consequently, co-expression will result in both Tm1-Tm2 interactions (which go to the PM) and Tm2-Tm2 interactions (which remain in the ER). It is also important to remember, from earlier studies, that co-expression of 8NTm1C1/GFP and GFP/8Tm2C2 results in forskolin- and Ca\(^{2+}\)-stimulable AC activity in intact HEK 293 cells, a property of AC8 that requires membrane cholesterol (7, 10). Therefore, taken together, these results indicate that the two transmembrane cassettes of AC8 are responsible for broad PM localization, whereas the intracellular C1 and C2 domains are necessary for localization to lipid rafts.

**DISCUSSION**

Co-localization of signaling partners is an increasingly encountered device that appears to have evolved to enhance selectivity and efficiency in signaling pathways. Two major strategies are possible: one (rather passive) the co-localization of partners to discrete domains of the PM and the other (more active) direct protein-protein interactions. In relation to signal transduction cascades, lipid rafts (35) have been proposed to
play a central role in the organization and efficient functioning of signaling pathways (3, 4, 24, 36–38). However, not all membrane-associated proteins involved in signal transduction are found in lipid rafts (7, 26, 39). ACs reside both in raft and nonraft domains. The Ca^{2+}-sensitive ACs (AC3, AC5, AC6, and AC8) are enriched in raft domains, whereas Ca^{2+}-insensitive ACs (AC2 and AC7) reside in other regions of the PM (3, 4, 7, 9, 26). This specific PM localization is believed to enable Ca^{2+}-sensitive ACs to respond to CCE, since disruption of lipid rafts by the cholesterol-extracting agent, methyl-β-cyclodextrin, ab-

**Fig. 8.** The TMDs of AC8 are not responsible for plasma membrane lipid raft targeting. HEK 293 cells transfected with the indicated GFP-tagged AC8 constructs containing the individual transmembrane cassettes of AC8 minus the C1 and C2 domains were lysed and prepared for sucrose equilibrium density gradient centrifugation as described under "Experimental Procedures". Fractions (lanes 3–10) were resolved by SDS-PAGE and immunoblotted with antibodies raised against caveolin (cav) and GFP (α-GFP). The same cells were fixed and processed for immunofluorescent confocal imaging. A, the N terminus and the first transmembrane cassette of AC8 (8Ntm1/GFP). B, the second transmembrane cassette of AC8 (GFP/8Tm2). C, co-expression of 8Ntm1/GFP and GFP/8Tm2.
lates the regulation of AC8 and AC6 by CCE (7, 9). However, nothing is known about the elements of AC structures responsible for targeting to lipid rafts.

As a prelude to investigating the determinants of AC raft localization, we confirmed that expressed AC5 and AC7 are found in separate identifiable PM domains in HEK 293 cells. There are two commonly used procedures to isolate lipid rafts. One uses cold extraction with nonionic detergents and relies on the insolubility of lipid rafts, due to close packing of saturated acyl chains and stabilization via cholesterol interdigitation. The second method does not use detergents but relies on sonication to break the PM into small fragments. Initially, the nondetergent method of raft enrichment was employed to investigate the raft localization of AC. When expressed in HEK 293 cells, AC5 was clearly enriched in lipid rafts, confirming previous results with endogenous AC5/AC6 in cardiac myocytes (24) and AC5 expressed in insect Sf9 cells (40). Certain internal organelles (e.g. Golgi) also contain lipid rafts, so we felt that it was important to use confocal analysis to confirm the PM localization of AC5 (25). Using the same methodologies, AC7 was detected only in nonraft bulk PM, confirming earlier studies based only on gradient fractionation (7). This clear difference between AC5 and AC7 allowed us to tackle the identification of regions that might be responsible for the discrete targeting of AC5 to lipid rafts by employing chimeric AC5-AC7 constructs.

A recently described and intuitively reasonable hypothesis for the targeting of proteins to rafts suggests the interaction between specific membrane proteins and cholesterol-sphingolipid complexes, termed lipid shells. These protein-lipid struc-
tures are thought to be stable and target the protein to preexisting rafts (41). Such an interaction may depend on specific residues within the TMDs of integral membrane proteins. Consequently, the TMDs of AC5 were considered as potentially important in raft targeting. Surprisingly, when the two TMD cassettes of AC7 were substituted for the corresponding domains of AC5, the resulting AC5 hybrid still occurred in lipid rafts. This finding indicates that interactions between the TMDs and raft lipids are unlikely to be involved in AC5 targeting. Since the TMDs of AC5 and AC7 are similar in hydrophathy profile but only 24% similar at the amino acid level, it appears that the major role of the TMDs is in assembling the catalytic domain from the component C1a and C2a domains, as suggested from earlier studies (10, 11).

Deletion of the N terminus of AC5 did not affect its targeting to rafts (Fig. 2). This finding reinforces a similar conclusion upon deletion of the N terminus of AC8 (7). Thus, only the cytoplasmic C1 and C2 domains can mediate AC5 targeting to rafts.

Since the C1a and C2a regions of ACs, which together form the catalytic domain, are most conserved among AC isoforms, the quite divergent C1b and C2b domains seemed likely candidates to control raft targeting. Exchanging the C1b domain of AC5 with the C1b domain from AC7 generated a construct that was restricted to the ER. One potential reason for the deficit in processing from the ER to the PM is that the C1b domain of AC7 is 21 amino acids longer than in AC5, which might impede intramolecular interactions between the TMDs that are required for PM targeting (10). Alternatively, the construct may be retained within the ER through the unmasking of a specific ER retention signal, although no obvious ER retention sequences were apparent in the sequence.

The C2b domain is also poorly conserved between AC isoforms. Deleting the C2b region of AC5 prevented membrane association, and the construct was detected only in the pelleted fraction at a lower than expected molecular weight. This implies that the C2b region is important for correct processing of AC5, deletion of the C2b domain appearing to initiate protein degradation, perhaps involving lysosomes, which would occur in the pelleted fraction. By contrast, deleting the C2b region of AC7 did not affect localization to the bulk PM fraction, which implies that the nonconserved C2b domains do not fulfill identical roles for individual ACs. Although deleting the C2b domain of AC5 produced only a degraded product, this does not exclude the possibility that the C2b domain is involved in raft targeting. When the C2b domain of AC7 replaced that of AC5, the expressed construct displayed the expected molecular weight but was not localized to the PM; this suggests that the C2b domain of AC7 is not sufficient to allow bulk membrane PM localization of AC5. The reciprocal construct, in which the C2b domain of AC7 was exchanged for that of AC5, allowed the examination of whether the C2b domain of AC5 was sufficient to take AC7 to lipid rafts. This construct did show partial localization to the buoyant membrane fraction but was predominantly present in nonraft membranes. Thus, the C2b region of AC5 is not itself sufficient to restrictively target AC5 to rafts; other domains must cooperate in the process.

Finally therefore, whether the combined, nonconserved C1b and C2b regions of AC5 were sufficient for raft targeting was investigated. AC7 containing only the C1b and C2b domains of AC5 occurred as a degraded product, which led us to conclude that full-length C1 and C2 domains are a minimum requirement for raft localization of AC5.

The isolation of lipid rafts by the nondetergent method has been suggested to produce a less selective representation of genuine raft proteins when compared with rafts isolated by cold Triton X-100 (4). This may be because the use of sonication alone to separate rafts from bulk membrane could allow nonraft lipids and proteins to remain associated (36). Therefore, we were reassured in confirming our earlier conclusions upon preparing rafts from purified PMs using cold Triton X-100. Although rafts prepared by detergents may permit membrane mixing through preferential solubilization of individual leaflets of the bilayer (42), the two independent methods of raft isolation used presently strongly suggest that the C1 and C2 cytoplasmic loops, and not the TMDs, underlie raft localization of AC5.

The importance of the C1 and C2 domains in raft localization was underscored by the finding that these domains were also responsible for the localization of AC8 to buoyant membrane domains. Co-expression of the two transmembrane cassettes of AC8, in the absence of the C1 and C2 domains, yields a tight complex at the PM, as determined by fluorescence resonance energy transfer and co-immunoprecipitation (10). Interestingly, fractionation revealed that this complex was broadly distributed at the PM but was most prominent in the bulk membrane fraction. Thus, as with AC5, the TMDs of AC8 are not sufficient to ensure raft localization. When the two halves of AC8 (i.e. 8NTm1C1/GFP and GFP/8Tm2C2) were expressed separately, both displayed an intracellular localization. However, co-expression revealed both halves of AC8 in buoyant membrane fractions. It is also significant that the co-expressed half-molecules of AC8 form a Ca2+-stimulable AC in intact cells (10). Given that AC8 requires membrane cholesterol for efficient in vivo stimulation by Ca2+, the two halves of AC8 must interact in PM lipid rafts (7). It would thus appear that both AC5 and AC8 are localized to lipid rafts through their respective C1 and C2 domains.

The present findings that the specific membrane targeting of AC5 and AC8 to lipid rafts appears to rely on protein-protein interactions governed by the cytoplasmic regions may at first seem counterintuitive. Although in some cases TMDs are implicated in lipid raft membrane localization (27, 28), not all integral membrane proteins are targeted through these domains (36, 43, 44). For example, CD20 a component of store-operated calcium entry in B-cells depends on cytoplasmic residues for raft localization (45). One protein that may target AC5 to rafts is caveolin. The muscle-specific caveolin-3 isoform and AC5/6 co-precipitate (24, 26, 40), and AC5 has been shown to interact with the caveolin scaffolding domain of caveolin-1 (46). However, caveolin cannot be solely responsible for lipid raft localization, since AC5 occurs in the buoyant membrane fraction from HEK 293 cells that lack caveolin-1 and -3 (24). Since ACs are now considered to participate in higher order macromolecular signaling complexes (3, 47, 48), this could provide an additional mechanism in targeting to lipid rafts (1). For example, AC and L-type calcium channels are closely associated in both neurons and myocytes (47, 49). Since L-type channels localize to rafts, this may in part contribute to the localization of additional proteins, including Ca2+-sensitive ACs (4). It is conceivable that a similar scenario occurs in nonexcitable cells, since targeting of Ca2+-sensitive ACs to raflike domains is an absolute requirement for responsiveness to CCE (7, 50–53). The molecular identity of the CCE channels remains elusive, although mammalian homologues of the Drosophila transient receptor potential proteins are candidates, some of which are found in rafts (54, 55). Thus, it is conceivable that a direct or indirect interaction between Ca2+-sensitive ACs and raft-localized CCE channels is the mechanism for AC targeting to rafts.

For the present, it is clear that the C1 and C2 domains of both AC5 and AC8 are not only involved in the formation of the
catalytic core but are also critical both in terms of PM targeting and protein handling. Whether these same domains underlie raft targeting of other Ca$^{2+}$-sensitive ACs remains to be determined.

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