Targeting of Protein Kinase Cα to Caveolae

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Abstract. Previously, we showed caveolae contain a population of protein kinase Cα (PKCα) that appears to regulate membrane invagination. We now report that multiple PKC isoenzymes are enriched in caveolae of unstimulated fibroblasts. To understand the mechanism of PKC targeting, we prepared caveolae lacking PKCα and measured the interaction of recombinant PKCα with these membranes. PKCα bound with high affinity and specificity to caveolae membranes. Binding was calcium dependent, did not require the addition of factors that activate the enzyme, and involved the regulatory domain of the molecule. A 68-kD PKCα-binding protein identified as *sdr* (serum deprivation response) was isolated by interaction cloning and localized to caveolae. Antibodies against *sdr* inhibited PKCα binding. A 100–amino acid sequence from the middle of *sdr* competitively blocked PKCα binding while flanking sequences were inactive. Caveolae appear to be a membrane site where PKC enzymes are organized to carry out essential regulatory functions as well as to modulate signal transduction at the cell surface.

THE protein kinase C (PKC)[1] family of phospholipid-dependent kinases are important regulators of growth, differentiation, and gene expression (8, 22). Based on the requirements for activation, the 12 mammalian PKC isoenzymes can be grouped into three categories (10): PKCα, βI, βII, and γ require calcium, phosphatidylserine (PS), and diacylglycerol (DAG) for activity; PKCε, δ, η, σ, and μ require PS and DAG; and PKCξ, ι, and λ need only PS. All isoenzymes have similar catalytic domains but differ in the structure of their regulatory domains. The intramolecular interaction between a 17–amino acid–long "pseudosubstrate" and the catalytic site may be a critical step in controlling the activity of many of these enzymes (5).

Most cells express multiple isoforms of PKC, and each has a specific set of functions (5). These isoenzymes, however, display little substrate specificity in in vitro assays. Therefore, other mechanisms must govern the specific function of each isoenzyme in the cell. One way to achieve specificity is by targeting individual isoenzymes to select locations in the cell (18), using high-affinity interactions between the enzyme and a subcellular compartment. The isoenzyme could be constitutively present in the target compartment or recruited there after the cell receives a stimulus. A variety of PKC-binding proteins (10) and lipids (22) have been identified that might function to compartmentalize PKC isoenzymes.

One place on the plasma membrane where PKCα appears to be a resident protein is caveolae (24, 25). Both cell fractionation and immunogold labeling of whole plasma membranes show that PKCα is highly concentrated in caveolae of unstimulated cells (25). Despite the presence of many different resident and migratory proteins in this domain (14), a 90-kD protein is the major PKCα substrate detected in intact cells as well as isolated caveolae (25). Phosphorylation in vitro occurs in the absence of activators such as DAG or PS (25), suggesting the enzyme is constitutively active when located in this compartment. The uptake of molecules by caveolae is linked to PKCα kinase activity (25), so the enzyme may play a key role in regulating the internalization of caveolae. Therefore, a mechanism must exist for directing PKCα to caveolae and regulating substrate specificity at this site. We now report that caveolae isolated from Rat-1 cells display a Ca^{++}-dependent, high-affinity PKCα binding activity that may be involved in targeting the enzyme to this domain. Using interaction cloning together with immunolocalization and a competitive binding assay, we have identified a protein component of this binding site as serum deprivation response protein (Sdr) (7).

Materials and Methods

Materials

Fetal calf serum was from Hazleton Research Products, Inc. (Lenexa, KS). DME, trypsin-EDTA, penicillin/streptomycin, and OptiPrep were

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1. *Abbreviations used in this paper*: DAG, diacylglycerol; MBP, maltose-binding protein; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PS, phosphatidylserine; sdr, serum deprivation response; RD, regulatory domain.

from GIBCO BRL (Gaithersburg, MD). Percoll was from Pharmacia Biotech (Piscataway, NJ). EGF was from CalBiochem (San Diego, CA). Human recombinant PKCα and PKCε were from PanVera Corporation (Madison, WI). ^{125}I-radiolabeled streptavidin with specific activity of 20–40 μCi/μg and ECL reagent were obtained from Amersham Corp. (Arlington, IL). Antibodies were obtained from the following sources: anti–caveolin-1 mAb IgG, anti–caveolin-1 polyclonal antibody IgG, anti-PKCα, -PKCε, -PKCλ IgGs (mAb), anti-RACK1 IgG (mAb), and anti–integrin β3 IgG (mAb) were from Transduction Laboratories (Lexington, KY); peroxidase-conjugated anti–mouse IgG and anti–rabbit IgG were from Organon Teknika (West Chester, PA); biotinylated goat anti–mouse IgG was from Vector Laboratories (Burlingame, CA); and TRITC-goat anti–mouse IgG [H+L] and FITC-goat anti–rabbit IgG [H+L] were from Zymed Laboratories Inc. (South San Francisco, CA). Polyclonal anti-*sdr* peptides were produced by standard methods. The PKCα pseudosubstrate peptide (RFARKGALRQKNVHENKN) was synthesized by University of Texas Southwestern Medical Center Polymer Core Facility. Immulon I Removawell 96-well plates were purchased from Dynatech Laboratories (Chantilly, VA). Immobilon transfer nylon was from Millipore (Bedford, MA). Crystalline bovine serum albumin and phorbol-12-myristate-13-acetate (PMA) were from Sigma Chemical Co. (St. Louis, MO). 1,1,1-trichloroethane was from Aldrich Chemical Co., Inc. (Milwaukee, WI).

Methods

Cell Culture. Rat-1 cells (6×10^5) were seeded in 100-mm-diam dishes and grown in 10 ml of DME supplemented with 10% (vol/vol) fetal calf serum for 4 d. Cells were then incubated for 24–48 h in DME without serum before each experiment. Normal human fibroblasts were cultured on coverslips as previously described (6).

Isolation of Caveolae Fractions. Detergent-free caveolae fractions were prepared by the method of Smart et al. (26). All steps were carried out at 4°C. Cells were collected by scraping in 5 ml of ice-cold buffer A (0.25 M sucrose, 1 mM EDTA, 20 mM tricine, pH 7.8, with or without 1 mM $CaCl_2$) and pelleting at 1,400 *g* for 5 min. After douncing, the postnuclear supernatant fraction was obtained, layered on top of 23 ml of 30% Percoll solution prepared in buffer A, and centrifuged at 84,000 *g* for 30 min (model Ti60 rotor; Beckman Instruments, Fullerton, CA). The plasma membrane band was collected and sonicated. The sonicate was mixed with 2 ml of 50% OptiPrep prepared in buffer B (0.04 M sucrose, 1 mM EDTA, 20 mM tricine, pH 7.8, with or without 1 mM $CaCl_2$) to make a 23% OptiPrep solution. The mixture was placed on the bottom of a centrifuge tube (model SW 41; Beckman Instruments) and overlaid with a linear 20 to 10% OptiPrep gradient (designated OptiPrep 1). After centrifugation at 52,000 *g* for 90 min in a swinging bucket rotor (model SW 41; Beckman Instruments), fractions were either analyzed directly (700 μl/fraction), or the top 5 ml of the gradient (fractions 1–7) was collected, mixed with 4 ml of 50% OptiPrep, overlaid with 2 ml of 5% OptiPrep in buffer A, and centrifuged at 52,000 *g* for 90 min (designated OptiPrep 2). An opaque band located just above the 5% interface was collected and designated the caveolae membrane fraction. Pooled fractions 8–14 from OptiPrep 1 were designated as the noncaveolae membrane fraction.

Electrophoresis and Immunoblots. Each sample was concentrated by TCA precipitation and washed in acetone. Pellets were suspended in Laemmli sample buffer (12), heated at 95°C for 3 min, and loaded onto 12.5% SDS polyacrylamide gel using the method of Laemmli (12). The separated proteins were transferred to nylon supports. The nylon was blocked in buffer C (20 mM Tris, pH 7.5, 137 mM NaCl, 0.5% Tween-20) containing 5% dry milk for 1 h at room temperature. Primary antibodies were diluted in buffer C containing 1% dry milk and incubated with the nylon samples for 1 h at room temperature. The nylon was washed four times for 10 min each in buffer C plus 1% dry milk and incubated with the appropriate HRP-labeled anti-IgG for 1 h at room temperature. The nylon was then washed, and the bands were visualized by enhanced chemiluminescence.

PKC Binding to Caveolae. PKCα binding to caveolae was carried out using either a solid phase or a solution assay. The solid phase radioimmune assay was modified from the method of Zhang et al. (28). Immulon I Removawell strips were washed twice with distilled water. Either caveolae or noncaveolae membranes isolated in the absence of calcium (3 μg) or BSA (3 μg) in 50 μl of buffer A were air dried to the bottom of each well. The coated wells were washed quickly three times with 250 μl of buffer D (25 mM Hepes, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mg/ml glucose, 0.1 mM EDTA, 1 mM DTT, 1 mg/liter leupeptin, 1 mg/liter pepstatin A) containing 1 mg/ml heat-denatured BSA, incubated with buffer D containing 2 mg/ml heat-denatured BSA for 45 min at room temperature, and washed three times with 250 μl of buffer D plus 1 mg/ml heat-denatured BSA. The indicated PKC mixtures (100 μl) were added and incubated for 30 min at the indicated temperature. The wells were washed rapidly seven times at 4°C with 250 μl of buffer D plus 1 mg/ml heat-denatured BSA. Each sample was fixed with 250 μl of 3% paraformaldehyde in buffer D for 30 min at room temperature. The amount of PKCα bound to EDTA-stripped membrane was determined by radioimmunoassay as previously described (28) using anti-PKCα (1 μg/ml), biotinylated goat anti–mouse IgG (2 μg/ml), and ^{125}I-streptavidin (2 μCi/ml).

To measure binding in solution, 15 μg of freshly isolated caveolae or noncaveolae membrane fractions were incubated in a polyallomer centrifuge tube (Beckman Instruments) for 30 min in the presence of purified PKCα (5 nM) under the indicated conditions. After the incubation, the sample was chilled at 4°C for 10 min and centrifuged for 60 min at 100,000 *g* to separate the membrane (pellet). The pellet was rinsed gently with buffer A, and 30 μl of Laemmli sample buffer was added. The sample was heated at 95°C for 3 min and loaded onto 12.5% SDS polyacrylamide gels. PKCα was detected by immunoblotting using mAb anti-PKCα IgG.

Isolation of sdr. Interaction cloning (4) was used to isolate a 68-kD PKCα-binding protein designated as clone 34. Analysis of the sequence showed that clone 34 was identical to a previously cloned protein known as *sdr* (7). Clone 34/*sdr* cDNA was ligated in frame into the pTrc (InVitrogen, Carlsbad, CA) or pQE (Qiagen, Chatsworth, CA) bacterial expression vector to produce recombinant His-tagged fusion proteins. The expressed sequences corresponding to polypeptides containing amino acids 1–168, 145–250, and 250–417 were purified by nickel-nitrilotriacetic acid chromatography according to the manufacturer's instructions. The purified peptides were used to raise antisera in rabbits. Antisera were purified by affinity chromatography using the expressed sequences coupled to Sepharose.

ELISA Assay. Fragments of clone 34/*sdr* containing residues 1–168, 145–250, or 250–417 (2.8 μg/ml in PBS, 100 μl per well) were bound to individual wells of a 96-well dish, and the wells were blocked with BSA (2% in PBS). PKCα (20 ng of recombinant PKCα) or RDα (60 ng of recombinant maltose-binding protein [MBP] fused to RDα) were added to the wells in buffer E (PBS plus 0.1 mg/ml BSA, 1 mM EGTA, 0.466 mM $CaCl_2$, and 2.1 mM $MgCl_2$). Reactions were incubated for 2 h at room temperature. Where indicated, PS (2 μg/ml) was included in the buffer. Wells were rinsed with buffer E and incubated with either PKCα-specific mAb M4 or anti-MBP polyclonal IgG for 1 h (New England Biolabs, Boston, MA) followed by the appropriate secondary antibody conjugated to HRP for 1 h, all in PBS plus 1 mg/ml BSA. Bound antibody was detected by adding 12 pmol/well of the substrate 2,2′azino-di[3 ethylbenzthiazoline sulfonate] in PBS and incubating for 15–60 min. Reaction was quantified by measuring the absorbance at 405 nm. Nonspecific binding of PKCα, and MBP-RDα was determined using BSA-blocked wells that did not contain peptides. PS did not influence nonspecific binding. Total bound PKCα or MBP-RDα corresponds to the amount of antibody binding to wells coated with 20 ng PKCα or 60 ng of MBP-RDα alone.

Indirect Immunofluorescence. Normal human fibroblasts and Rat-1 cells grown on glass coverslips were washed quickly with buffer F (100 mM sodium phosphate, pH 7.6, containing 3 mM KCl and 3 mM $MgCl_2$) and then fixed in methanol/acetic acid/1,1,1 trichloroethane (60:10:30) for 20 min. Cells were quickly rinsed three times with 50% methanol followed by three times with buffer F. Cells were incubated with buffer F containing 0.8% bovine serum albumin for 30 min, followed by buffer F containing 20 μg/ml mAb anti–caveolin-1 plus a 1:10 dilution of anti-*sdr* IgG for 60 min. Finally, cells were incubated for 60 min in the presence of buffer F containing 20 μg/ml goat anti–mouse IgG conjugated to TRITC and 20 μg/ml goat anti–rabbit IgG conjugated to FITC. After incubation, cells were washed and mounted in a 2.5% solution of 1,4-diabicyclo-(2,2,2) octane. All incubations were at room temperature. Samples were photographed using a Zeiss Photomicroscope III (Thornwood, NY).

Other Assays. Protein concentrations were determined using Bio-Rad Bradford assay (Hercules, CA).

Results

Previously, we localized PKCα to caveolae of MA104 cells using a cell fractionation scheme that depends on the partial insolubility of caveolae in Triton X-100 at 4°C (25). To avoid potential artifacts associated with the use of detergents, in the current studies we isolated caveolae from purified plasma membranes by flotation on OptiPrep gradi-

ents (26). The first (OptiPrep 1) of the two gradients used in the purification separates light membranes rich in the caveolae marker caveolin-1 from the bulk plasma membrane protein. The second gradient (OptiPrep 2) further purifies the caveolae from the top seven fractions of the first gradient. The standard buffer for this cell fractionation procedure contains 1 mM EGTA. Immunoblots of each fraction (total protein load) from OptiPrep 1 gradients of Rat-1 cell plasma membranes showed low levels of PKCα in the caveolin-rich (*caveolin*) light fractions (Fig. 1 *A*, *PKCα*, lanes *1–7*). Little PKCα was detected in the heavier fractions (lanes *8–14*) that had the bulk of the plasma membrane protein (Fig. 1 *D*, *squares*), although these fractions contained all of the detectable integrin β3 (lanes *8–14*).

Association of PKC with Caveolae

To determine if the EGTA had stripped away PKCα from caveolae during the isolation, we prepared cell fractions using the same buffer with 1 mM Ca^{++} added (Fig. 1 *B*). Under these conditions, the caveolin-rich fractions contained a much higher concentration of PKCα. Since all the protein in each fraction was loaded on the gel, the majority of the PKCα we detected was in these fractions (compare lanes *1–7* with lanes *8–14*). The protein profile (Fig. 1 *D*, *diamonds*) as well as the distribution of caveolin-1 and integrin $1\beta_3$ were unchanged. If the cells were preincubated in the presence of PMA for 20 min before fractionation, the light membrane fractions had similar levels of PKCα, even though calcium was not in the isolation buffer (fractions *1–7*, compare Fig. 1, *B* and *C*, *PKCα*). PKCα was not detected in the bulk membrane fractions under either condition (Fig. 1, *B* and *C*, lanes *10–14*). These results suggest PKCα is normally bound to caveolae through a calcium-sensitive interaction with resident molecules.

Other PKC isoforms were also found to be enriched in caveolae fractions (Fig. 2). PKCλ was concentrated in caveolae, but unlike PKCα, enrichment was stimulated by a lack of Ca^{++} in the isolation buffer (compare lanes *1* and *2*). This isoform was also enriched when cells were pretreated with PMA for 20 min (lane *3*). PKCε was enriched in the absence of Ca^{++} (lane *1*), but the presence of Ca^{++} slightly reduced the concentration (lane *2*). Pretreatment of cells with PMA increased the amount of PKCε in the caveolae fraction relative to other treatments (lane *3*). Thus, PKC isoenzyme types differ in the amount of calcium required to remain bound to caveolae membrane during isolation but share the ability to remain bound independently of calcium after cells are pretreated with PMA.

We used immunoblotting to measure the relative amount of PKCα in the cytosol, noncaveolae membrane (*NCM*), and caveolae membrane (*CM*) fractions after various isolation conditions (Fig. 3). When Ca^{++} was in the isolation buffer, PKCα was enriched in caveolae (compare lane *12* with *11*) but not noncaveolae fractions (compare lane *7* with *6*). The slight increase in PKCα concentration seen in the cytosol fraction under these conditions was within experimental variability (compare lane *1* with *2*). Both caveolae (lane *13*) and noncaveolae (lane *8*) fractions had similar low levels of PKCα when Mg^{++} was substituted for Ca^{++}. Exposing cells to PMA for 20 min caused an apparent increase in the amount of PKCα in the

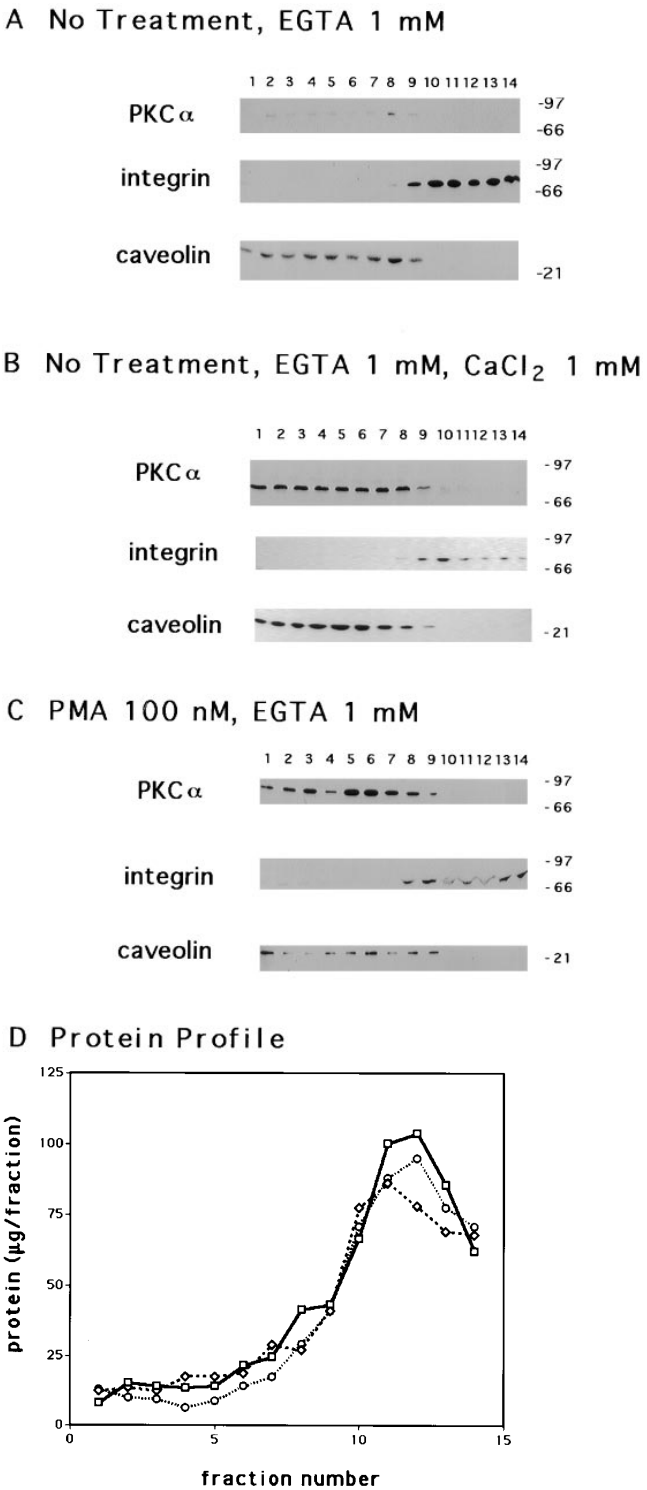

Figure 1. Effects of EGTA (*A*), calcium (*B*), and PMA pretreatment (*C*) on the presence of PKCα in caveolae membrane fractions. Rat-1 cells grown 24 h in the absence of serum were incubated in the presence (*C*) or absence (*A* and *B*) of 100 nM PMA for 20 min at 37°C before fractionating sonicated plasma membranes in the presence (*B*) or absence (*A*) of 1 mM $CaCl_2$ on an OptiPrep 1 gradient. Equal volume fractions were taken from the top (fraction *1*) to bottom (fraction *14*), separated by electrophoresis, and immunoblotted with either monoclonal anti-PKCα IgG, anti–integrin β3 IgG, or anti–caveolin-1 IgG. The protein profiles (*D*) for each gradient (*squares*, gradient A; *diamonds*, gradient B; *circles*, gradient C) were similar.

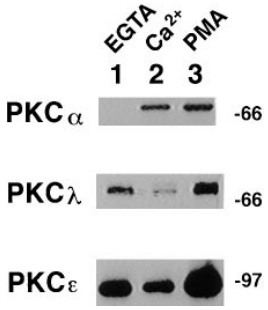

Figure 2. Effects of EGTA (lane *1*) or calcium (lane *2*) in the isolation buffer or pretreatment of cells with PMA (lane *3*) on the presence of PKC isoenzymes in caveolae membrane fractions. Rat-1 cells grown 24 h in the absence of serum were incubated in the presence (lane *3*) or absence (lanes *1* and *2*) of 100 nM PMA for 20 min at 37°C before preparing caveolae membrane fractions in the presence (lane *2*) or absence (lanes *1* and *3*) of 1 mM $CaCl_2$. Equal protein loads (5 μg) were separated by electrophoresis and immunoblotted with the indicated anti-PKC IgG.

caveolae fraction relative to isolation in the absence of Ca^{++} (compare lane *14* with *11*) without changing the amount in either the cytosol (lane *4*) or the noncaveolae (lane *9*) fractions. By contrast, extended exposure of cells to PMA caused a reduction in the cytosolic level of PKCα (compare lane *5* with *1*) and completely eliminated the protein from the caveolae fractions (compare lane *15* with *14*).

Binding of PKCα to Caveolae

The lack of detectable PKCα in the bulk plasma membrane fractions rich in integrin β3 (Fig. 1 *B*), even though we loaded the total protein in each fraction (up to 100 μg/lane in fractions *11* and *12*) on the gel, suggests PKCα has a specific affinity for caveolae. We used a solid phase assay to determine if caveolae were able to bind PKCα (Fig. 4). Caveolae and noncaveolae membranes were isolated in the absence of Ca^{++} so that PKCα was not present (see Fig. 2). Equal amounts of caveolae (Fig. 4 *A*, bars *1–6*) and noncaveolae (bar *7*) membrane protein were air dried on the bottom of 96-well plates and assayed for PKCα binding. When caveolae membranes were incubated in the presence of the complete binding mixture (1.5 nM PKCα, 1 mM Ca^{++}, 30 μM PS, 100 μM ATP) at 37°C for 30 min (bar *1*), significant amounts of PKCα bound to caveolae membranes. By contrast, very little PKCα bound to noncaveolae membranes (bar *7*). Binding to caveolae was prevented by removing either PKCα (bar *2*) or Ca^{++} (bar *3*) from the mixture. Mg^{++} could not substitute for Ca^{++} (bar *4*), and PS was not required (bar *5*). Finally, PKCα did not bind to caveolae when the incubation was carried out at 4°C (bar *6*).

PKCα binding to caveolae membranes in the solid phase assay was saturable (Fig. 4 *B*, *squares*). Half-maximal binding occurred at ~0.5 nM PKCα, suggesting a high-affinity interaction with the membrane. Binding of PKCα to noncaveolae membranes (*circles*) was no greater than binding to dishes coated with albumin (*diamonds*).

We could also detect PKCα binding to caveolae using a solution assay (Fig. 4 *C*). Caveolae and noncaveolae membranes were prepared and incubated in solution with the indicated mixtures. At the end of each incubation, the membranes were recovered by centrifugation, processed for gel electrophoresis, and immunoblotted with either anti–caveolin-1 IgG (*caveolin*) or anti-PKCα IgG (*PKCα*). The association of PKCα with the pelleted caveolae fraction was dependent on the presence of PKCα (compare lanes *1* and *2*), Ca^{++} (compare lanes *2* and *3*), and temperature (compare lanes *2* and *6*), but not PS (compare lanes *2* and *5*). Binding was not detected if noncaveolae membrane was substituted for caveolae (compare lanes *2* and *7*) or if Ca^{++} was replaced with Mg^{++} (compare lanes *2* and *4*).

The solid phase assay was used to define further the requirements for PKCα binding to caveolae membranes. We showed in Fig. 1 that the calcium requirement for PKCα association with isolated caveolae was lost when cells were incubated in the presence of PMA before caveolae isolation. By contrast, the addition of PMA to the in vitro binding assay mixture had no effect on PKCα binding to isolated caveolae (Fig. 5 *A*). The amount of PKCα bound was the same in the presence or absence of PMA (compare bars *1–3*). Furthermore, PMA did not promote PKCα binding to caveolae when calcium was removed from the incubation mixture (compare bars *4* and *5* with *2* and *3*). No binding was detected when noncaveolae membranes (bar *6*) or albumin (bar *7*) were substituted for caveolae. In other experiments, we found that PMA did not stimulate PKCα binding to noncaveolae membranes (data not shown).

We originally added ATP to the incubation mixture because PKCα contains an ATP-binding domain that might be required for interacting with caveolae. Fig. 5 *B* shows, however, that ATP was not required for PKCα binding (compare bars *1* and *2*). GTP also had no effect on binding (data not shown). We still did not detect binding to caveo-

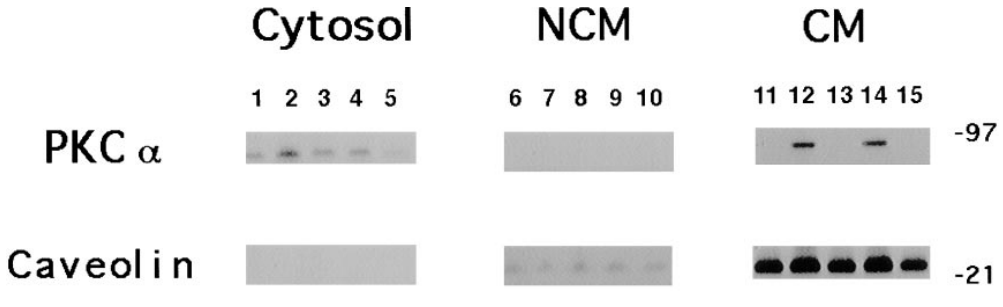

Figure 3. Comparative blotting activity of PKCα in cytosol, noncaveolae membrane (*NCM*), and caveolae membrane (*CM*) fractions. Rat-1 cells were grown in the absence of serum for 24 h and either incubated in the presence of PMA for 20 min (lanes *4*, *9*, and *14*) or 16 h (lanes *5*, *10*, and *15*) or not pretreated. Fractions were prepared in the continuous presence of EGTA with either 1 mM $CaCl_2$ (lanes *2*, *7*, and *12*), 2.5 mM $MgCl_2$ (lanes *3*, *8*, and *13*), or nothing (lanes *1*, *4–6*, *9–11*, *14*, and *15*) added to the buffer. Samples of cytosol (50 μg), noncaveolae membrane (*NCM*, 5 μg), and caveolae membrane (*CM*, 5 μg) were separated by gel electrophoresis and immunoblotted with antibodies directed against PKCα and caveolin-1.

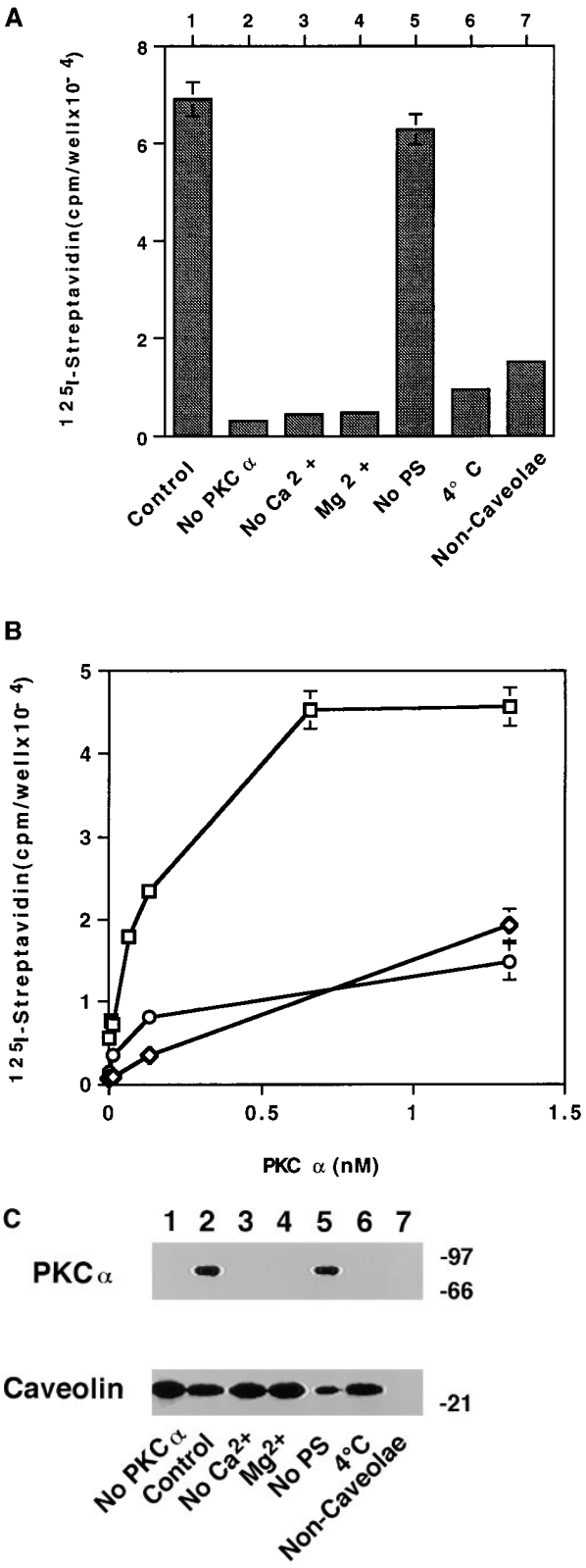

Figure 4. PKCα binding to isolated caveolae and noncaveolae membrane fractions using either a solid phase (*A* and *B*) or solution (*C*) assay. The indicated membrane fractions were prepared form Rat-1 cells grown 24 h in the absence of serum using standard methods. (*A*) Membranes (3 μg) were air dried into individual wells of a 96-well plate and incubated in the presence of 100 μl of either complete buffer 37°C (*Control*) or 4°C or complete buffer at 37°C with the indicated modifications (no Ca^{++}, Mg^{++}, no PS, or no PKCα). The amount of bound PKCα was measured using an indirect radioimmune assay as described in Materials and Methods. (*B*) Individual wells of a 96-well plate containing 3 μg of either caveolae membrane (*squares*), noncaveolae membrane (*circles*), or BSA (*diamonds*) were incubated with the indicated amounts of PKCα before assaying for bound enzyme as described. Values are the average of triplicate measurements ± the standard deviation. (*C*) Isolated caveolae membrane (lanes *1–6*) or noncaveolae membrane (lane *7*) was incubated in the presence of 5 nM purified PKCα (reaction volume 300 μl) for 30 min at 37°C (lanes *1–5* and *7*) or 4°C (lane *6*) using either complete buffer (*control*) or complete buffer with the indicated modifications (no Ca^{++}, Mg^{++}, no PS, or no PKCα). After the incubation, the samples were chilled, pelleted at 100,00 *g* for 60 min at 4°C, separated by gel electrophoresis, and immunoblotted with either anti-PKCα or anti–caveolin-1 IgG.

lae at 4°C (compare bars *3* and *4*) or to noncaveolae membranes (compare bars *5* and *6*) when ATP was removed from the incubation buffer. Also, the lack of PKCα binding to caveolae at 4°C did not change if PS was removed from the incubation mixture (data not shown).

Since Ca^{++} is required for PKCα binding but not ATP, the regulatory domain (RDα) of the molecule may mediate binding to caveolae. We compared the binding to caveolae membranes of recombinant forms of PKCα and RDα (amino acids 1–312). Caveolae (Fig. 6 *A*, bars *1–4*) and noncaveolae (bars *5* and *6*) membranes were incubated in the presence of 1.3 nM PKCα or 1.3 nM RDα. When Ca^{++} was in the buffer (compare bars *1* and *3*), equal amounts of either PKCα or RDα bound to caveolae membranes. Removal of Ca^{++} from the buffer (compare bars *2* and *4*) reduced binding to the level seen when noncaveolae membranes were substituted for caveolae (compare bars *2* and *4* with *5* and *6*). Further evidence for RDα-mediated binding is that PKCε, which contains a different regulatory domain that appears not to require calcium for association with caveolae (see Fig. 2), did not block PKCα binding to caveolae membranes even when present in >100-fold excess (Fig. 6 *B*, compare bars *1–5*).

Identification of a PKCα-binding Protein in Caveolae

Most likely, the high-affinity binding of PKCα to caveolae involves an interaction with a resident protein of caveolae. A candidate protein should bind PKCα in the presence of calcium, bind the regulatory domain of PKCα, and be concentrated in caveolae. Several PKC-binding proteins have been identified by probing expression libraries with recombinant PKC (called interaction cloning [10]). A protein isolated from such a screen with the required characteristics is clone 34. Clone 34 is a 68-kD protein identical in sequence to *sdr*, which was isolated from serum starved cells (7). In an overlay assay, clone 34/*sdr* bound the regulatory domain of PKCα only when calcium and PS were present (data not shown). We used a quantitative binding assay to localize the region of clone 34/*sdr* that contains the PKCα-binding domain (Fig. 7). Samples of histidine-tagged fusion protein containing either amino acids 1–168, 145–250, or 250–417 of clone 34/*sdr* were bound to individ-

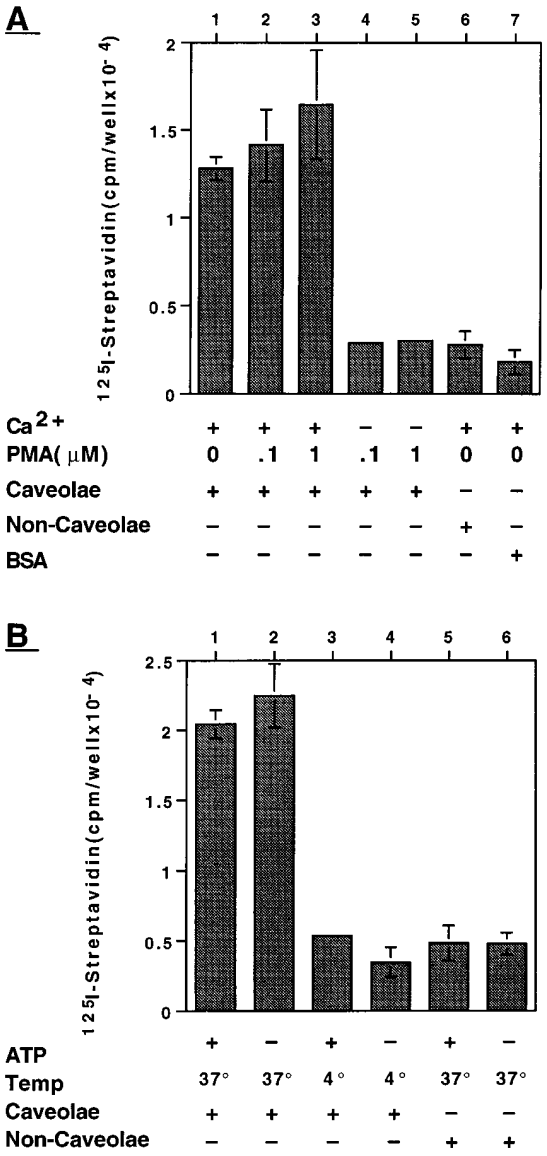

Figure 5. Neither PMA (*A*) nor ATP (*B*) was required for PKCα binding to caveolae. The solid phase binding assay was using the standard buffer with the indicated additions at 37°C as described in Fig. 4 *A*. Incubations were carried out in the presence (+) or absence (−) of calcium with the indicated concentration of PMA in the buffer. (*B*) Incubations were carried out in the presence (+) or absence (−) of ATP at the indicated temperature. Values are the average of triplicate measurements ± the standard deviation.

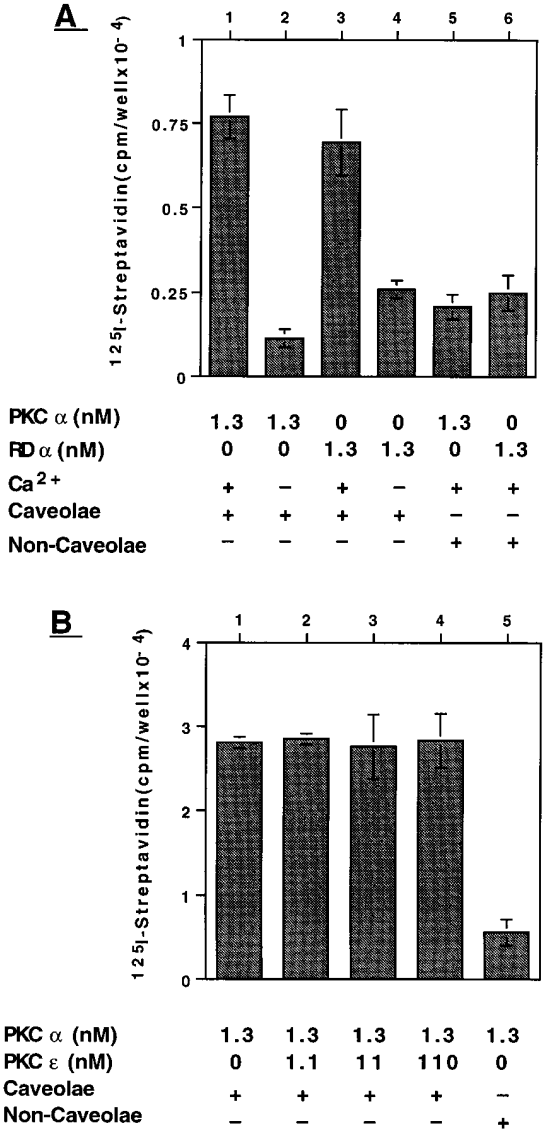

Figure 6. The regulatory domain of PKCα (*A*), but not intact PKCε (*B*), binds caveolae. The solid phase binding assay was using the standard buffer with the indicated additions at 37°C as described in Fig. 4 *A*. Incubations were carried out in the presence (+) or absence (−) of calcium with the indicated concentration of regulatory domain (*RDα*) or PKCα in the buffer. (*B*) Incubations were carried out using a complete buffer containing the indicated amounts of PKCα and PKCε. Values are the average of triplicate measurements ± the standard deviation.

ual wells of a 96-well plate. Wells were then incubated in the presence of either the full-length (PKCα) or the regulatory domain of PKCα (RDα) in the presence or absence of PS before assaying for the amount bound. Both PKCα (*left*) and RDα (*right*) bound peptide 145–250 in the presence (*hatched bars*) but not the absence of PS (*solid bar*). Neither PKCα nor RDα bound the other two peptides.

Fig. 8 shows the immunofluorescence colocalization of clone 34/*sdr* (*B*) and caveolin-1 (*A*) in a human fibroblast. Some anti–clone 34/*sdr* IgG staining had a perinuclear (*N*, nucleus) distribution characteristic of the Golgi apparatus. Staining was also prominent along the edges of the cell and in linear patches on the surface (*arrowheads*). The edge and surface patches colocalized with caveolin-1 (compare *arrowheads* between *A* and *B*). The mAb anti–caveolin-1 used to do the colocalization reacted poorly with Rat-1 cells. Nevertheless, when we used polyclonal anti–caveolin-1 IgG (*C*) and anti–clone 34/*sdr* IgG (*D*) on separate sets of cells, a similar edge staining (*arrowheads*) was evident in both sets. Immunoblots of total protein loads from Rat-1 cell OptiPrep 1 gradient fractions (*E*) showed that PKCα, clone 34/*sdr*, and caveolin-1 quantitatively cofractionated (fractions *1–8*). By contrast, another PKC-binding protein, RACK 1 (receptor for activated C

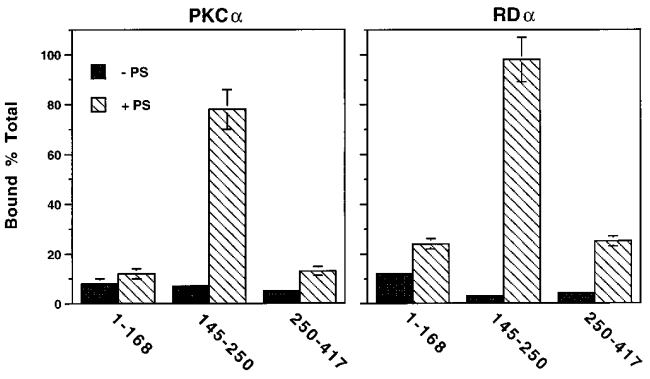

Figure 7. Localization of the PKCα-binding region of clone 34/*sdr*. The indicated peptides were bound to individual wells of a 96-well plate and incubated in the presence of either recombinant PKCα or an MBP–PKCα regulatory domain fusion protein (RDα) as described. The amount of each molecule bound was measured using an indirect immunoperoxidase detection assay. Each value is expressed as the percent of total PKCα or RDα bound to wells coated with the respective protein. Each value is the average of triplicate measurements ± the standard deviation.

kinase [19]), was primarily in the bulk plasma membrane fraction (fractions *9–14*).

We used the solid phase binding assay to see if anti–clone 34/*sdr* IgG blocked PKCα binding to caveolae (Fig. 9 *A*). Good binding was observed when caveolae fractions were incubated with the complete binding mixture (bar *1*). Addition of 15 μg of the affinity-purified anti–clone 34/*sdr* IgG to the incubation mixture reduced PKCα binding by ~50% (bar *2*). Increasing the concentration of the antibody did not further reduce binding. The same concentration of polyclonal anti–caveolin-1 IgG, by contrast, had no effect on PKCα binding (bar *4*). PKCα did not bind to noncaveolae membranes (bar *4*). These results suggest clone 34/*sdr* is a protein component of the PKCα-binding site.

A peptide competition assay provided additional evidence that clone 34/*sdr* was involved in PKCα binding to caveolae (Fig. 9 *B*). We used subsaturating concentrations of PKCα in a standard binding assay where each tested peptide was present in 100-fold excess. Compared with no additions (bar *1*), peptide 1–168 had no effect on PKCα binding (bar *2*). Peptide 145–250, by contrast, reduced binding to the level seen when noncaveolae membranes were substituted for caveolae (compare bars *3* and *6*). Peptide 250–417 did not inhibit binding (bar *4*). We also tested the effect of the PKCα pseudosubstrate peptide on binding (bar *5*). This peptide completely blocked binding (bar *5*). Therefore, we have localized peptide domains within both clone 34/*sdr* and PKCα that can interact during PKCα binding to caveolae membranes.

Discussion

PKCα Binding to Caveolae

Cell fractionation and immunocytochemistry have previously shown that PKCα is constitutively present in caveolae and that this is a major cell surface location for the enzyme (25). We used a solid phase binding assay that has successfully identified other membrane binding sites for cytosolic proteins (28) to determine if PKCα would bind to caveolae. PKCα bound with high affinity (binding was dependent on calcium) did not require the addition of either PMA, PS, or ATP, and only occurred at 37°C. PKCα did not bind to noncaveolae membranes, which contain >90% of the plasma membrane protein starting material. The same specific interaction with caveolae was also detected in a solution binding assay. Caveolae, therefore, exhibit a PKCα binding activity that may be responsible for targeting the enzyme to this compartment.

We found that caveolae contained other members of the PKC enzyme family. Fractions from untreated cells contained PKCα only when calcium was present and PKCλ only when calcium was absent from the isolation buffer. The presence of PKCε was not dependent on calcium, although this cation did appear to reduce the amount of enzyme in the fraction. The calcium concentration needed to retain the enzyme during isolation is a reflection of the cation requirement for PKC binding to caveolae. These results raise the possibility that local fluctuations in the concentration of calcium can regulate the amount of a PKC isoenzyme in caveolae. Calcium could function, therefore, as a regulatory switch that controls the isoenzyme composition of caveolae. This may be especially important at times when calcium entry occurs at caveolae (2).

PMA did not significantly increase the level of PKC in caveolae above that normally present when isolation was carried out under the correct calcium conditions for retention of the isoenzyme (Fig. 2, lane *3*). This suggests that PMA does not stimulate recruitment of cytosolic PKCs to caveolae but instead stabilizes the resident population of isoenzyme so it remains bound regardless of the concentration of calcium in the isolation buffer. This conclusion is supported by the finding that PMA did not induce binding of PKCα to either caveolae or noncaveolae membranes in vitro (Fig. 5 *A*).

Recombinant PKCα was used in all of the in vitro assays, so binding to isolated caveolae was not dependent on phosphorylation of the enzyme. Furthermore, the regulatory domain alone bound as well as the whole protein, and this region does not contain any of the phosphorylation sites thought to modulate the interaction of PKCα with the cytoskeleton (20). PMA was also not required for binding, nor did it block binding (Fig. 4 *A*), and calcium was required for retention during caveolae isolation. These are the characteristics of a binding site designed to recognize inactive, native PKCα within the cell and concentrate the enzyme at caveolae independently of the activation state of the cell. There may be binding sites specific for each of the major isoenzyme families. The specificity required to distinguish between isoenzyme families may be conferred by other PKC-binding proteins together with cofactors concentrated in caveolae. The PKC isoenzymes in caveolae are probably engaged in regulating essential cellular activities.

One activity that PKCα appears to regulate at this location is the internalization of caveolae (25). The phosphorylation of a 90-kD caveolae substrate occurs during invagination and sequestration of molecules by caveolae. Cells lacking PKCα do not have detectable enzyme in caveolae,

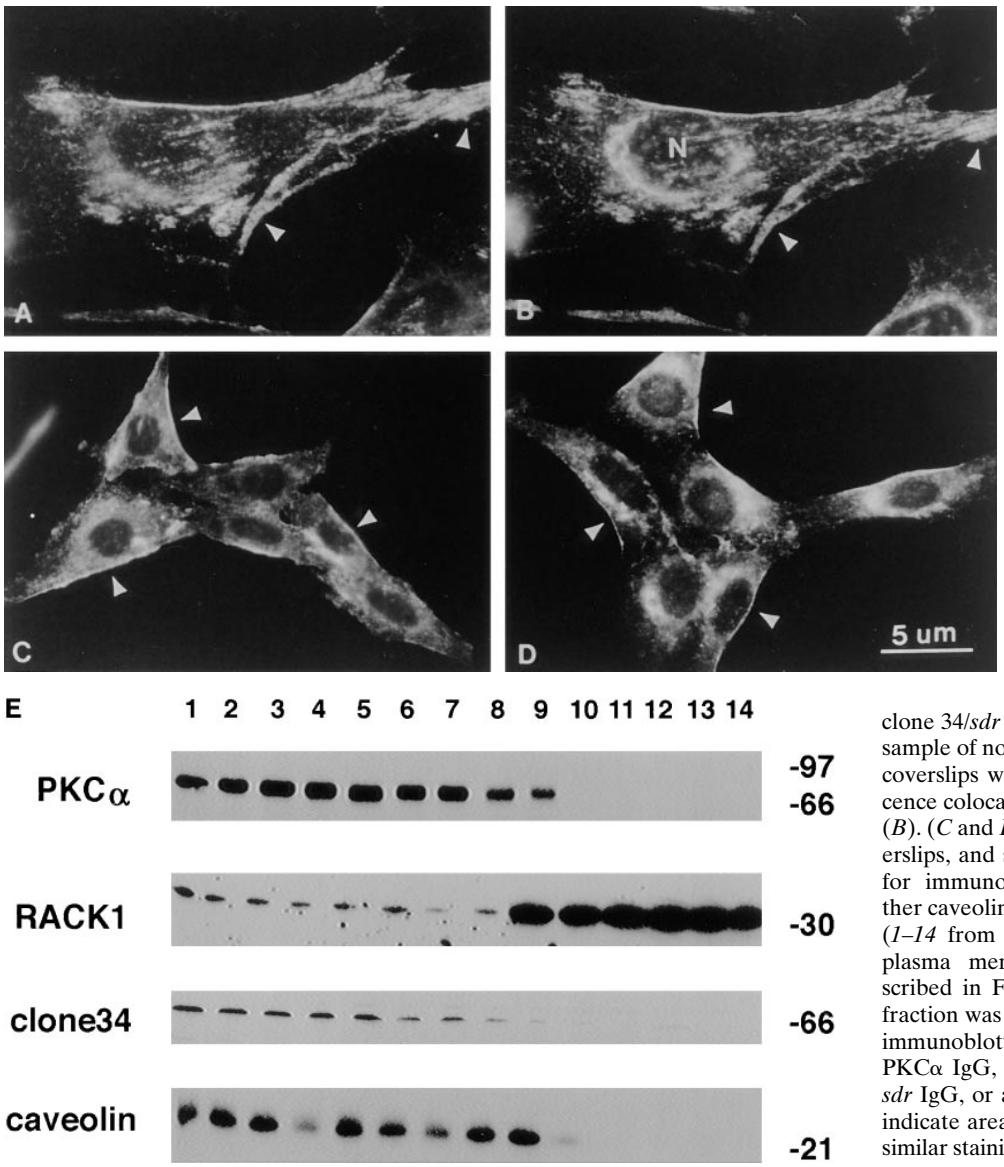

Figure 8. Immunofluorescence (*A–D*) and cell fractionation (*E*) localization of clone 34/*sdr* to caveolae. (*A* and *B*) The same sample of normal human fibroblasts grown on coverslips was processed for immunofluorescence colocalization of caveolin-1 (*A*) and *sdr* (*B*). (*C* and *D*) Rat-1 cells were grown on coverslips, and separate samples were processed for immunofluorescence localization of either caveolin-1 (*C*) or *sdr* (*D*). (*E*) OptiPrep 1 (*1–14* from the top) fractions of Rat-1 cell plasma membrane were prepared as described in Fig. 1. An equal volume of each fraction was separated by electrophoresis and immunoblotted with either monoclonal anti-PKCα IgG, anti-RACK1 IgG, anti–clone 34/*sdr* IgG, or anti–caveolin-1 IgG. Arrowheads indicate areas of colocalization (*A* and *B*) or similar staining patterns (*C* and *D*). Bar, 5 μm.

and both caveolae invagination and ligand internalization are blocked. Like many resident proteins of caveolae, the PKCα in this domain is normally resistant to solubilization by Triton X-100 at 4°C. After stimulation of histamine H_1 receptors, membrane-bound PKCα becomes detergent soluble, suggesting a change in its linkage to the caveolae membrane. Under these conditions, phosphorylation of the 90-kD substrate does not occur, and internalization of caveolae is inhibited. The binding activity we have detected may be essential for positioning PKCα to optimize the phosphorylation of this protein. Another outcome of binding is to localize PKC isoenzymes at a site where they can interact with multiple signaling pathways (2).

Localization of a PKCα-binding Protein to Caveolae

A number of PKC-binding proteins have been identified that could participate in targeting PKCα to caveolae (10, 18, 21), including caveolin (23). We focused our attention on clone 34/*sdr* because initial immunofluorescence examination suggested it was present in caveolae. Immunofluorescence and cell fractionation of Rat-1 cells clearly show that the majority of the plasma membrane clone 34/*sdr* is concentrated in caveolae. Clone 34/*sdr* was in caveolae fractions isolated without calcium even after PMA pretreatment of cells (data not shown). The binding of PKCα to both caveolae and purified clone 34/*sdr* requires calcium and the regulatory domain of PKCα. In addition, neither activity requires ATP or an activator such as PMA. Anti–clone 34/*sdr* IgG reduces PKCα binding by 50%, and a specific peptide (amino acids 145–250) within *sdr* competitively inhibits binding. These results suggest clone 34/*sdr* has a role in targeting PKCα to caveolae.

sdr was originally isolated from NIH 3T3 cells in a screen for RNA messages that are upregulated during serum deprivation (7). *sdr* contains a leucine zipper-like motif between amino acids 50 and 100 and two consensus sites for PKC phosphorylation. One of the phosphorylation sites (amino acids 229–250) is at the amino terminus of the *sdr* peptide that binds the regulatory domain of

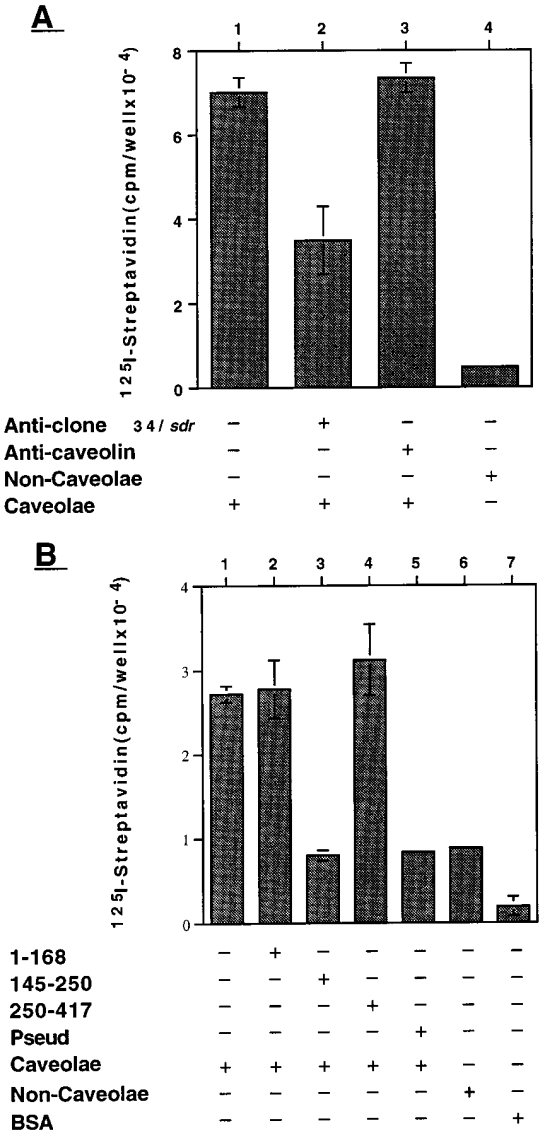

Figure 9. Anti–clone 34/*sdr* IgG (*A*) and specific clone 34/*sdr* peptides (*B*) block binding of PKCα to caveolae. Caveolae and noncaveolae membranes were prepared from Rat-1 cells, and the solid phase binding assay was used at 37°C to detect binding as described in Fig. 4 *A*. (*A*) Incubations were carried out in the presence (+) or absence (−) of affinity-purified anti–clone 34/*sdr* IgG or anti–caveolin-1 IgG, and both used a concentration of 15 μg/ml in the complete buffer system. (*B*) Recombinant PKCα (1.3 nM) was preincubated in the presence (+) or absence (−) of the indicated peptides from clone 34/*sdr* (130 nM) or pseudosubstrate (130 nM) in the complete buffer for 30 min at 37°C. Values are the average of triplicate measurements ± the standard deviation.

PKCα and blocks its binding to caveolae. SRBC (sdr-related gene product that binds C-kinase) (9) shares several similarities with *sdr*, including binding PS as well as the regulatory domain of PKC and phosphorylation by PKC. These two proteins belong to a class of molecules called STICKs (substrates that interact with C-kinase [10]). Each STICK may have a primary function in targeting a distinct set of PKC isoenzymes to specific locations in the cell. Interestingly, a fusion protein with cell transforming activity was isolated from colon cancer cells that consists of the first 184 amino acids of SRBC linked to c-Raf (27). Since activation of c-Raf takes place in caveolae (16), and a c-Raf containing the COOH-terminal consensus sequence for prenylation is constitutively active (13) in caveolae (17), the SRBC–Raf fusion protein may alter cell behavior by inappropriately targeting c-Raf to this membrane domain. If this is the case, then the first 184 amino acids of SRBC are predicted to contain a caveolae binding motif.

The targeting of PKCα to caveolae is probably more complex than a simple one-to-one interaction between the enzyme and *sdr*. Unlike caveolae, PKCα binding to purified *sdr* can occur at 4°C, requires PS, and is not blocked by the PKCα pseudosubstrate peptide. Caveolae could provide the needed PS, but it is hard to reconcile the other two differences if *sdr* acts alone. Caveolae membrane lipids, unlike surrounding regions of membrane, are in a liquid order phase owing to the high concentration of cholesterol and sphingomyelin (3). The phase properties of membrane lipids are temperature sensitive, raising the possibility that a higher lateral mobility of membrane proteins and lipids at 37°C is required for PKCα binding to caveolae. There also must be molecules in caveolae that concentrate the *sdr* itself because it does not contain any obvious membrane anchor. Whatever these interactions turn out to be, they probably influence the amount of PKCα in caveolae. Finally, the PKCα in caveolae is active (25), so DAG, a lipid species that is enriched in caveolae (15), is probably bound to this population of enzyme (22). The pseudosubstrate of the enzyme, therefore, may be free to interact with nearby molecules, which could account for why the pseudosubstrate peptide interfered with PKCα binding to caveolae. We conclude that a protein, or group of proteins, act coordinately in the proper lipid environment to attract PKCα to caveolae.

Compartmentalization of PKC Function by Caveolae

The finding that multiple PKC isoenzymes along with at least one known PKC-binding protein are concentrated in caveolae suggests this is a location where the signaling function of these molecules is compartmentalized. The combination of a unique membrane environment and a close physical association should enable caveolae PKC isoenzymes to perform unique functions that do not occur anywhere else in the cell. Some of these functions may be housekeeping in nature, such as controlling the invagination of caveolae. The proximity of these PKCs to other signaling molecules in this domain (1, 2), however, will naturally facilitate interactions that influence many different signaling events. The immediate goals are to identify caveolae-specific PKC functions and to determine the mechanism(s) used to organize these enzymes at this location on the cell surface. There may be a protein scaffold (11) that holds several isoenzymes in a PKC module, linking receptors with multiple targets through a kinase cascade (22). Molecules like *sdr* might function as linkers, adaptors, or switches that control interactions among the elements of this module.

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