Targeting of Protein Kinase Cα to Caveolae

Chieko Mineo,* Yun-Shu Ying,* Christine Chapline,‡ Susan Jaken,§ and Richard G.W. Anderson*

*Department of Cell Biology and Neuroscience, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9039; and ‡Adirondack Biomedical Research Institute, Lake Placid, New York 12946

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) 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, phosphatidylerine (PS), and diacylglycerol (DAG) for activity; PKCδ, η, ζ, and μ require PS and DAG; and PKCε, 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 Ca2+-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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from Gibco BRL (Gaithersburg, MD). Percoll was from Pharmacia Bio-
tech (Piscataway, NJ). EGF was from CalBiochem (San Diego, CA). Hu-
man recombinant PKCα and PKCγ were from PanViva (Madison, WI). 125I-
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, -PKCβ, -PKCδ IgGs (mAb), 
anti-integrin αvβ3 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 (model Ti60 rotor; Beckman Instruments, Fullerton, CA). The plasma 
was then washed, and the bands were visualized by enhanced chemilu-
mination 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-nitritotrptic acid 
chromatography according to the manufacturer’s instructions. The puri-
fied peptides were used to raise antiserum in rabbits. Antiserum was 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 recombi-
nant 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 
CaCl2, and 2.1 mM MgCl2). 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 F and incubated with either PKCα-specific 
MBP or anti-MBP polyclonal IgG for 1 h (New England Biolabs, Bos-
ton, 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-ethylbenzthiazo-
ilone sulfonate] in PBS and incubating for 15–60 min. Reaction was quanti-
fied 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 MgCl2) 
and then fixed in methanol/acetic acid/1,1-trichloroethane (40:10:30) 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) oct-
ane. All incubations were at room temperature. Samples were photo-
graphed 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-
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\(^{2+}\) 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 β3 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). PKCα was concentrated in caveolae, but unlike PKCα, enrichment was stimulated by a lack of Ca\(^{2+}\) 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\(^{2+}\) (lane 1), but the presence of Ca\(^{2+}\) 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, nonspecific plasma membrane (NCM), and caveolae membrane (CM) fractions after various isolation conditions (Fig. 3). When Ca\(^{2+}\) 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\(^{2+}\) was substituted for Ca\(^{2+}\). 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.
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 PKC\(\alpha\) (compare lane 5 with 1) and completely eliminated the protein from the caveolae fractions (compare lane 15 with 14).

**Binding of PKC\(\alpha\) to Caveolae**

The lack of detectable PKC\(\alpha\) in the bulk plasma membrane fractions rich in integrin \(\beta3\) (Fig. 1 B), even though we loaded the total protein in each fraction (up to 100 \(\mu\)g/lane in fractions 11 and 12) on the gel, suggests PKC\(\alpha\) has a specific affinity for caveolae. We used a solid phase assay to determine if caveolae were able to bind PKC\(\alpha\) (Fig. 4). Caveolae and noncaveolae membranes were isolated in the absence of Ca\(^{++}\) so that PKC\(\alpha\) 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 PKC\(\alpha\) binding. When caveolae membranes were incubated in the presence of the complete binding mixture (1.5 nM PKC\(\alpha\), 1 mM Ca\(^{++}\), 30 \(\mu\)M PS, 100 \(\mu\)M ATP) at 37\(^\circ\)C for 30 min (bar 1), significant amounts of PKC\(\alpha\) bound to caveolae membranes. By contrast, very little PKC\(\alpha\) bound to noncaveolae membranes (bar 7). Binding to caveolae was prevented by removing either PKC\(\alpha\) (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, PKC\(\alpha\) did not bind to caveolae when the incubation was carried out at 4\(^\circ\)C (bar 6).

PKC\(\alpha\) binding to caveolae membranes in the solid phase assay was saturable (Fig. 4 B, squares). Half-maximal binding occurred at ~0.5 nM PKC\(\alpha\), suggesting a high-affinity interaction with the membrane. Binding of PKC\(\alpha\) to noncaveolae membranes (circles) was no greater than binding to dishes coated with albumin (diamonds). We could also detect PKC\(\alpha\) 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-PKC\(\alpha\) IgG (PKC\(\alpha\)). The association of PKC\(\alpha\) with the pelleted caveolae fraction was dependent on the presence of PKC\(\alpha\) (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 PKC\(\alpha\) binding to caveolae membranes. We showed in Fig. 1 that the calcium requirement for PKC\(\alpha\) 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 had no effect on PKC\(\alpha\) binding to isolated caveolae (Fig. 5 A). The amount of PKC\(\alpha\) bound was the same in the presence or absence of PMA (compare bars 1-3). Furthermore, PMA did not promote PKC\(\alpha\) 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 PKC\(\alpha\) binding to noncaveolae membranes (data not shown).

We originally added ATP to the incubation mixture because PKC\(\alpha\) contains an ATP-binding domain that might be required for interacting with caveolae. Fig. 5 B shows, however, that ATP was not required for PKC\(\alpha\) binding (compare bars 1 and 2). GTP also had no effect on binding (data not shown). We still did not detect binding to caveolae membranes 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 \(\mu\)g), noncaveolae membrane (NCM, 5 \(\mu\)g), and caveolae membrane (CM, 5 \(\mu\)g) were separated by gel electrophoresis and immunoblotted with antibodies directed against PKC\(\alpha\) and caveolin-1.
of either complete buffer at 37°C did not change if PS was removed from the incubation mixture (data not shown).

Since Ca\(^{2+}\) is required for PKC\(\alpha\) binding but not ATP, the regulatory domain (RD\(\alpha\)) of the molecule may mediate binding to caveolae. We compared the binding to caveolae membranes of recombinant forms of PKC\(\alpha\) and RD\(\alpha\) (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 PKC\(\alpha\) or 1.3 nM RD\(\alpha\). When Ca\(^{2+}\) was in the buffer (compare bars 1 and 3), equal amounts of either PKC\(\alpha\) or RD\(\alpha\) bound to caveolae membranes. Removal of Ca\(^{2+}\) 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 RD\(\alpha\)-mediated binding is that PKC\(\alpha\), which contains a different regulatory domain that appears not to require calcium for association with caveolae (see Fig. 2), did not block PKC\(\alpha\) binding to caveolae membranes even when present in >100-fold excess (Fig. 6 B, compare bars 1–5).

Identification of a PKC\(\alpha\)-binding Protein in Caveolae

Most likely, the high-affinity binding of PKC\(\alpha\) to caveolae involves an interaction with a resident protein of caveolae. A candidate protein should bind PKC\(\alpha\) in the presence of calcium, bind the regulatory domain of PKC\(\alpha\), 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 PKC\(\alpha\) 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 PKC\(\alpha\)-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-

buffer at 37°C with the indicated modifications (no Ca\(^{2+}\), Mg\(^{2+}\), no PS, or no PKC\(\alpha\)). The amount of bound PKC\(\alpha\) 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 PKC\(\alpha\) 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 PKC\(\alpha\) (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\(^{2+}\), Mg\(^{2+}\), no PS, or no PKC\(\alpha\)). 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-PKC\(\alpha\) or anti–caveolin-1 IgG.
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 ATP at the indicated temperature. Values are the average of triplicate measurements ± the standard deviation.

Figure 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
PKC or an MBP–PKC sdr. The indicated peptides were bound to individual wells of a (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 PKC bound to wells coated with the respective protein. Each value is the average of triplicate measurements ± the standard deviation.

Figure 7. Localization of the PKCα-binding region of clone 344/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.

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 PKCα only when calcium was absent from the isolation buffer. The presence of PKCζ 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,
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 H1 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 PKCα. This motif is required for PKCα binding and sdr localization to caveolae. 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.

**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.
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 transform-
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