Localization of Platelet-derived Growth Factor-stimulated Phosphorylation Cascade to Caveolae*

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Previously we showed that interleukin 1β stimulates the conversion of sphingomyelin to ceramide in the caveolae fraction of normal human fibroblasts. The ceramide, in turn, blocked platelet-derived growth factor (PDGF) stimulated DNA synthesis. We now present evidence that the PDGF receptor initiates signal transduction from caveolae. Cell fractionation and immunocytochemistry show caveolae to be the principal location of PDGF receptors at the cell surface. Multiple caveolae proteins acquire phosphorytrosine when PDGF binds to its receptor, but the hormone appears to have little effect on the tyrosine phosphorylation of non-caveolae membrane proteins. Five proteins known to interact with the phosphorylated receptor were found to be highly enriched in caveolae membrane. PDGF caused the concentration of three of these proteins to significantly increase in the caveolae fraction. Finally, PDGF stimulated the association of a 190-kDa phosphoprotein with the caveolae marker protein, caveolin. Therefore, ceramide may modulate PDGF receptor function directly in caveolae.

The PDGFβ receptor belongs to a family of growth factor receptors that transmit information across the membrane by stimulating the phosphorylation of specific effectors such as PI 3-kinase, non-receptor tyrosine kinases, protein-tyrosine phosphatases, and various adapter molecules (1). Activation of the receptor requires the binding of PDGF, the dimerization of the receptor, and receptor tyrosine phosphorylation (2, 3). The phosphorylated receptor then attracts effector molecules containing both SH2 and SH3 domains where they become active in reassembling to various cellular compartments. Shortly after ligand binding, the ligand-receptor complex is internalized and degraded (4). Internalization appears to be mediated by clathrin-coated pits (5, 6).

The exact location on the cell surface where the PDGFβ receptor initiates its signaling cascade has not been determined. The receptor may do this either from a random position on the plasma membrane or from a specific membrane domain. Recently, plasmaemal membrane caveolae have been identified as a site where several PDGF receptor effector molecules are concentrated (7–9). This raises the possibility that the receptor is also located in this compartment.

Caveolae were first identified as distinctive membrane invaginations on the cell surface. The shape of the membrane suggested they were involved in endocytosis. Indeed, caveolae appear to be involved in the uptake of bulk proteins from the blood (10), the internalization of small molecules such as folate by potocytosis (11), and the internalization of glycolipid binding toxins (12). Internalization of caveolae exhibitize molecules in a cyclic process involving the progressive invagination of the plasma membrane, followed by the formation of a closed, vesicle-like compartment, and the return of the vesicle to the plasma membrane. This cycle is hormonally regulated (13) as well as sensitive to pharmacological agents that block either the initial invagination of the membrane (14) or the return of the vesicle from the cell interior (15).

Cells may also use caveolae to compartmentalize signal transduction at the cell surface (11, 16). Recently we reported that IL-1β stimulates the conversion of sphingomyelin to ceramide only in the caveolae membrane of normal human fibroblasts (17). The ceramide, in turn, blocks PDGF-stimulated DNA synthesis in these cells. The modulation of PDGF signaling could occur directly in caveolae or at a remote location in the cell. To distinguish between these possibilities, we first needed to know where the PDGF-stimulated phosphorylation cascade originates. We now present evidence this occurs in caveolae.

EXPERIMENTAL PROCEDURES

Materials

PDGF (BB), insulin, EGF, acidic FGF, anti-PDGβ receptor polyclonal, anti-phosphotyrosine monoclonal, anti-MAP kinase monoclonal, anti-Src monoclonal, and agarose-conjugated anti-phosphotyrosine monoclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-caveolin, anti-PI 3-kinase, anti-Syp, anti-Nck, and anti-Shc monoclonal antibodies were obtained from Transduction Laboratories (Lexington, KY). Anti-caveolin polyclonal (K617) was raised in our laboratory. PVDF membrane was from Millipore. ECL Western blotting detection reagents were from Amersham (Buckinghamshire, UK). OptiPrep was purchased from Accurate Chemical & Scientific Corp. (Westbury, NY). Protein A-Sepharose CL4B beads were from Pharmacia Biotech Inc.

Methods

Cell Culture—Normal human fibroblasts (18) were cultured to confluence in minimal essential medium supplemented with penicillin and 10% fetal bovine serum and then incubated in minimal essential medium containing 200 μg/ml bovine serum albumin for 12 h. Caveolae isolation and immunoprecipitation were done on confluent cells cultured in 150-mm plates. Immunoelectron microscopy and immunofluorescence experiments were done on cells cultured on coverslips.

Caveolae Isolation—Caveolae were isolated by the method of Smart et al. (19). Briefly, confluent normal human fibroblasts were collected in hypotonic buffer and dounced 20 times on ice. Plasma membrane (PM) was isolated in a 30% Percoll gradient from postnuclear supernatant.
immunofluorescence staining was concentrated in patches on the cell surface (Fig. 1C) as other regions of membrane. Only background labeling was seen on membranes exposed to the nonimmune rabbit IgG (Fig. 1B) or an irrelevant mAb (Fig. 1C). A separate set of cells was processed to localize the PDGF receptor (D) and caveolin (E) in the same cell. F, immunogold co-localization of PDGF receptor (5 nm gold) and caveolin (10 nm gold) in isolated fibroblast plasma membranes. Normal human fibroblasts were grown in the absence of serum for 12 h. Upper plasma membranes were attached to Formvar-coated grids and incubated in the presence of a mixture containing polyclonal anti-PDGFβ receptor IgG and mAb anti-caveolin IgG (Cavelin), B–E, indirect immunofluorescence co-localization of PDGF receptors and caveolin. Normal human fibroblasts grown in the absence of serum for 12 h were processed for immunofluorescence. Two different cells were processed using either a nonimmune rabbit IgG (B) or an irrelevant mAb (C). A separate set of cells was processed to localize the PDGF receptor (D) and caveolin (E) in the same cell. F, immunogold co-localization of PDGF receptor (5 nm gold) and caveolin (10 nm gold) in isolated fibroblast plasma membranes. Normal human fibroblasts were grown in the absence of serum for 12 h. Upper plasma membranes were attached to Formvar-coated grids and incubated in the presence of a mixture containing polyclonal anti-PDGFβ receptor IgG and mAb anti-caveolin IgG, immunogold localization on isolated plasma membrane using a nonimmune rabbit IgG as the primary antibody.

**RESULTS**

Localization of PDGFβ Receptor—A detergent-free method was used to purify caveolae from quiescent normal human fibroblasts maintained in the absence of serum for 12 h. A standard protocol was established where 5 µg of either the postnuclear supernatant (PNS), the plasma membrane (PM), the non-caveolae membrane (NCM), or the caveolae membrane (CM) was separated by electrophoresis and transferred to PVDF membrane for immunoblotting. When these fractions were immunoblotted with anti-PDGF receptor IgG (Fig. 1A), a single band of ~185 kDa was present in the PNS (PDGFR, lane 1). The same band was more intense in the membrane fraction but not detectable at all in the non-caveolae membrane fraction (PDGFR, compare lanes 2 and 3). By contrast, an extremely intense PDGF receptor band was present in the caveolae fraction (PDGFR, lane 4), indicating a high degree of concentration relative to the plasma membrane. Exactly the same pattern was seen when the samples were immunoblotted with the caveolae marker, anti-caveolin IgG (Cavelin, Fig. 1A).

We used immunochemistry to verify the location of the receptor. Normal human fibroblasts grown on coverslips were incubated in either media alone, 30 ng/ml PDGF (BB), 10 µg/ml insulin, 50 ng/ml EGF, or 20 ng/ml acidic FGF for 15 min. Cells were washed twice with ice-cold phosphate-buffered saline, lysed with TETN (25 mM Tris-HCl, pH 7.5, 5 mM EDTA, 1% Triton X-100) made in 150 mM NaCl plus 60 mM octyl glucoside (Calbiochem), protease inhibitors, and phosphatase inhibitors before being placed on a rocking platform for 40 min at 0°C. The cell lysate was centrifuged at 13,000 × g for 10 min, precleared with preblocked protein A-Sepharose CL-4B beads (Pharmacia) for 4 h at 4°C, and incubated for 12 h at 4°C either with polyclonal anti-caveolin IgG or the preimmune IgG from the same rabbit plus preblocked protein A-Sepharose CL-4B beads (500 µg of protein/5 µg of IgG/50 µl of beads). The beads were washed twice with TETN in 500 mM NaCl, twice with TETN in 250 mM NaCl, and twice with TE (10 mM Tris-HCl, pH 7.5, and 5 mM EDTA) for 5 min each at 4°C. In other experiments, protein samples were immunoprecipitated with agarose-conjugated anti-phosphotyrosine monoclonal antibody (designated Anti-Tyr(P) IgG) and anti-PDGF receptor IgG (designated anti-PDGF-R) for 40 h at 4°C using the same conditions. Proteins were eluted from beads with SDS sample buffer, separated by SDS-polyacrylamide gel electrophoresis, transferred to PVDF membrane, and blotted with the indicated antibody.

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PDGF Stimulates phosphorylation of Caveolin Proteins—
The localization of the PDGF receptor to caveolae suggested that the receptor phosphorylation cascade might begin at this site. The first step in this sequence is the phosphorylation of the receptor. Caveolae were prepared from unstimulated (lanes 1–4, Fig. 2A) or PDGF-stimulated (lanes 5–8, Fig. 2A) cells. The standard fractions were immunoblotted either with anti-PDGF receptor IgG (PDGFR) or mAb anti-phosphotyrosine IgG (PY-PDGFR). The receptor was highly concentrated in the caveolar fraction from unstimulated cells, but the anti-Tyr(P) IgG gave only a weak signal, indicating that little of this receptor was phosphorylated. Cells incubated in the presence of PDGF for 15 min, by contrast, had the same amount of receptor in the caveolar fraction but a band of the same molecular weight as the receptor reacted strongly with the anti-Tyr(P) IgG. There were no phosphotyrosine-positive bands of this size detected in the non-caveolar membrane, and only a faint band was evident in the plasma membrane fraction (lane 6).

The kinetics of PDGF-dependent receptor phosphorylation are shown in Fig. 2B. Caveolae were isolated at various times after PDGF was added to the culture media. Before the addition of PDGF, the receptor band did not react with the anti-Tyr(P) IgG (0 min, PY-PDGFR). After 5 min of incubation in the presence of PDGF, strong reactivity appeared (5 min, PY-PDGFR). The phosphorylated receptor remained in caveolae until, after 30 min of incubation, the concentration began to decline (30 min). Coincident with the nearly complete loss of the receptor by 120 min (PDGFR), little phosphoprotein was detected in the caveolar fraction at this time (PY-PDGFR). PDGF-stimulated phosphorylation of the PDGF receptor was accompanied by the tyrosine phosphorylation of a number of proteins in the caveolar fraction (Fig. 3A). Caveolae (CM) and non-caveolae (NCM) fractions were isolated from cells after exposure to either media alone (0 min) or PDGF for various times (5–120 min). Fractions were immunoblotted with anti-Tyr(P) IgG and exposed for either 30 s (Exp. 1) or 5 min (Exp. 2). In the absence of PDGF, only one band of 63 kDa reacted with the anti-Tyr(P) IgG (0 min, CM). The addition of PDGF, however, stimulated a complex pattern of phosphorylation in the caveolar fraction. At least 7 bands could be resolved (189, 97, 78, 73, 43, 38, 23 kDa) after just 5 min of incubation. An additional three bands appeared after 15–30 min (126, 86, and 73 kDa). Between 60 and 120 min, many of these bands disappeared, commensurate with the loss of the PDGF receptor from the caveolar fraction (see Fig. 2B). Nevertheless, there were five bands that persisted. We could only detect a single reactive band in the non-caveolar fractions (NCM) during this interval, even though 5 times more protein was loaded in each lane.

Immunoblotting was used to determine if any intermediates in the PDGF receptor cascade were present in the caveolar fractions of either unstimulated or stimulated cells (Fig. 3B). Normal human fibroblasts were incubated in the presence (+) or absence (−) of PDGF for 15 min before various fractions were prepared for immunoblotting. In the molecular weight range of 60,000–75,000, we found the tyrosine phosphatase Syp (Syp), the adapter molecule Shc 66 (Shc), and pp60src kinase (Src) to be enriched in caveolae of unstimulated cells. Nck (47 kDa) and MAPK (42 kDa) were also enriched, as was the noncatalytic subunit of PI 3-kinase (85 kDa). After the addition of PDGF, there appeared to be an increase in the amount of Syp, Shc, and MAPK in the caveolar fraction (compare − with +; Syp, Shc, MAPK) while the concentration of the other enriched proteins remained unchanged.

Anti-caveolin IgG Co-precipitates a Phosphoprotein—The caveolar resident protein caveolin has been found to interact with heterotrimeric G proteins (22) as well as a yet to be identified 30-kDa tyrosine phosphoprotein (23, 24). We used an immunoprecipitation protocol to see if any of the PDGF recep-
tor substrates also interacted with caveolin (Fig. 4A). A whole cell extract was prepared from cells incubated in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of PDGF for 15 min. The extracts were processed for immunoprecipitation using either polyclonal anti-caveolin IgG (lanes 2 and 4) or a preimmune IgG (lanes 1 and 3). The proteins were separated and immunoblotted with anti-Tyr(P) IgG (top gel) or anti-caveolin IgG (bottom gel). A single protein of ~190 kDa reacted with the anti-Tyr(P) IgG in anti-caveolin immunoprecipitates from PDGF-treated (lane 4) cells but not immunoprecipitates from control cells (lane 2). This band was not present when the anti-caveolin IgG was replaced with a preimmune IgG (lanes 3). Only the immunoprecipitates obtained with the anti-caveolin IgG (lanes 2 and 4) contained immunodetectable caveolin (caveolin).

The appearance of the 190-kDa phosphoprotein was specific for PDGF (lane 2, Fig. 4B). We were unable to detect the phosphoprotein in cells exposed to either insulin (compare lane 3 with lane 2), EGF (compare lane 4 with lane 2), or acidic FGF (compare lane 5 with lane 2) even though equal amounts of caveolin were immunoprecipitated (middle panel). On the other hand, all of the peptide hormones stimulated an increase in the amount of tyrosine-phosphorylated MAPK (bottom gel, compare lane 1 with lanes 2–5) immunoprecipitated with an anti-Tyr(P) IgG, indicating they were active in these cells.

The phosphoprotein is approximately the same size as the PDGF receptor (Fig. 5A). Therefore, we separated on polyacrylamide gels samples of anti-caveolin (lanes 4 and 5) and preimmune (lanes 2 and 3) immunoprecipitates from cells incubated either in the presence (lanes 3 and 5) or absence (lanes 2 and 4) of PDGF and immunoblotted with anti-PDGF receptor IgG (Fig. 5A). We could not detect a reactive band in these samples even though the anti-Tyr(P) IgG reacted with the 190-kDa protein specifically in samples from PDGF-stimulated cells (Anti-PY, compare lane 4 with 5). PDGF receptor was detected in isolated caveolae from stimulated cells (Anti-PDGR, lane 1) together with a strong anti-Tyr(P) positive band (Anti-PY, lane 1). A close comparison of the anti-Tyr(P) IgG reactive bands in lanes 1 and 5 indicates that the two phosphoproteins have slightly different molecular weights.

We also could not detect caveolin in anti-PDGF receptor IgG immunoprecipitates (Fig. 5B). Strong immunoblot reactivity with an anti-Tyr(P) IgG was present in samples from PDGF-stimulated cells (Anti-PY, lane 4) but caveolin could not be detected (Anti-Caveolin, lane 4). In the same experiment, we...
detected a time-dependent association of the 190-kDa phosphoprotein with caveolin. Anti-caveolin immunoprecipitates were prepared from cells that had been incubated in the presence of PDGF for various times (lanes 5–9) and blotted with either anti-Tyr(P) IgG (Anti-PY) or anti-caveolin IgG (Anti-Caveolin). Initially there was no phosphoprotein in the immunoprecipitate (Anti-PY, lane 5). After 5 min, a reactive band was present (lane 6) and remained associated with caveolin until 120 min (lane 9). The loss of the 190-kDa protein from anti-caveolin immunoprecipitates coincided with the migration of PDGF receptor out of caveolae (Fig. 2).

The intensity of the anti-Tyr(P) IgG-positive, 190-kDa band in the caveolin fraction was markedly less than the band of similar molecular weight in the anti-caveolin IgG immunoprecipitate (Fig. 5B, compare lanes 4 and 6). The two bands also appeared to have slightly different molecular weights. Nevertheless, we wanted to determine directly that PDGF stimulated receptor phosphorylation in the caveolin fraction (Fig. 5C). Samples (30 μg of protein) of postnuclear supernatant (lanes 1 and 5), plasma membrane (lanes 2 and 6), non-caveolae membrane (lanes 3 and 7), and caveolin membrane (lanes 4 and 8) were prepared from cells that had been incubated in the presence (lanes 5–8) or absence (lanes 1–4) of PDGF. Anti-Tyr(P) IgG immunoprecipitates were prepared from these samples, separated by gel electrophoresis, and immunoblotted with anti-PDGF receptor IgG. PDGF receptor was not detected in any of the fractions from control cells (lanes 1–4). After PDGF treatment, however, the anti-Tyr(P) IgG immunoprecipitated PDGF receptors from the postnuclear supernatant (lane 5) and the plasma membrane (lane 6). Immunoprecipitates of non-caveolae membranes yielded little PDGF receptor (lane 7), but there was a very intense band in the caveolin membrane (lane 8). These results further emphasize the select location of the PDGF receptor on the surface of fibroblasts.

**DISCUSSION**

Light and electron microscopic immunocytochemistry, as well as immunoblots of membrane fractions, show PDGF receptors to be concentrated in caveolae. We cannot determine with certainty that this is the exclusive location of these receptors at the cell surface. Nevertheless, we did not detect tyrosine phosphorylation of non-caveolae membrane proteins in stimulated cells, and five molecules known to interact with PDGF receptor during signal transduction were, like the receptor, clearly enriched in caveolae. The receptors began to migrate out of caveolae after 1 h of exposure to PDGF. Coincidently, the number of phosphotyrosine proteins in this fraction declined. This suggests that the termination of the tyrosine phosphorylation signal is coupled to the loss of the receptor from caveolae. The most parsimonious interpretation of these results is that caveolae are major sites of PDGF-stimulated signalling transduction.

We also found that a 190-kDa phosphoprotein interacts with caveolin in cells exposed to PDGF. Neither this protein, nor any other phosphoprotein, was detected in immunoprecipitates from cells exposed to other hormones. This protein is not the PDGF receptor. Nevertheless, it is the third protein found to interact with caveolin. This suggests that caveolin, in addition to functioning in cholesterol transport (21), plays a role in organizing caveolae proteins during signal transduction (22).

These results further confirm a role for caveolae in processing information at the cell surface (16). So far we have localized four other signaling events to caveolae: regulation of caveolae internalization by histamine (13), EGF-dependent activation of Raf-1, endothelial nitric-oxide synthase activity (26), and IL-1β-stimulated ceramide production (17). In addition, a variety of hormone receptors and signal transducing molecules appear to be enriched in caveolae (27). The presence of all these molecules at one location most likely is required for the proper integration of multiple signaling events occurring simultaneously in each cell. Integration of PDGF and IL-1β signaling through caveolin appears to be a good model system for studying how this occurs. The effect of ceramide on PDGF-stimulated phosphorylation of effector molecules can now be determined directly. This will set the stage for understanding how ceramide function is altered in damaged caveolae from transformed cells (25). The conversion of sphingomyelin to ceramide or the interaction of ceramide with the PDGF receptor might be impaired in these cells, leading to uncontrolled cell proliferation.

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