Tyrosine-phosphorylated caveolin is a physiological substrate of the low M(r) protein-tyrosine Phosphatase

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 22, Issue of June 1, pp. 18849–18854, 2001
Printed in U.S.A.

Tyrosine-phosphorylated Caveolin Is a Physiological Substrate of the Low M_r Protein-Tyrosine Phosphatase*

Received for publication, January 25, 2001
Published, JBC Papers in Press, March 14, 2001, DOI 10.1074/jbc.M100705200

Anna Caselli, Maria Letizia Taddei, Giampaolo Manao, Guido Camici‡, and Giampietro Ramponi

From the Dipartimento di Scienze Biochimiche, Università di Firenze, 50134 Firenze, Italy

Low M_r phosphotyrosine-protein phosphatase is involved in the regulation of several tyrosine kinase growth factor receptors. The best characterized action of this enzyme is on the signaling pathways activated by platelet-derived growth factor, where it plays multiple roles. In this study we identify tyrosine-phosphorylated caveolin as a new potential substrate for low M_r phosphotyrosine-protein phosphatase. Caveolin is tyrosine-phosphorylated *in vivo* by Src kinases, recruits into caveolae, and hence regulates the activities of several proteins involved in cellular signaling cascades. Our results demonstrate that caveolin and low M_r phosphotyrosine-protein phosphatase coimmunoprecipitate from cell lysates, and that a fraction of the enzyme localizes in caveolae. Furthermore, in a cell line sensitive to insulin, the overexpression of the C12S dominant negative mutant of low M_r phosphotyrosine-protein phosphatase (a form lacking activity but able to bind substrates) causes the enhancement of tyrosine-phosphorylated caveolin. Insulin stimulation of these cells induces a strong increase of caveolin phosphorylation. The localization of low M_r phosphotyrosine-protein phosphatase in caveolae, the *in vivo* interaction between this enzyme and caveolin, and the capacity of this enzyme to rapidly dephosphorylate phosphocaveolin, all indicate that tyrosine-phosphorylated caveolin is a relevant substrate for this phosphatase.

Caveolin is a small integral membrane protein and the principal component of caveolae membrane domains *in vivo* (1). Caveolae are plasma membrane-attached vesicular organelles that have a characteristic diameter in the 50–100-nm range (2, 3). Although caveolae are present in most cells, they are particularly abundant in terminally differentiated cells such as adipocyte, endothelial, and skeletal muscle cells. Recent studies have revealed the presence of at least three mammalian caveolin subtypes, caveolin-1, caveolin-2, and caveolin-3, with different tissue distribution (see Ref. 4, and references therein). Different from the role originally proposed, *i.e.* as a protein that organizes the caveolae structure and accumulates many signaling molecules, a new role for caveolin as a regulator of transmembrane signaling has been suggested by recent data. Several research teams have demonstrated that caveolin is able to recognize and recruit into caveolae, and to regulate the activities of several proteins involved in cellular signaling cascades, such as the heterotrimeric G-proteins, Src kinases, nitric-oxide synthase, epidermal growth factor, and platelet-derived growth factor receptors, as well as protein kinase C (see Ref. 4, and references therein). The interaction between caveolin and other proteins is mediated through a short stretch of the membrane-proximal region (or caveolin scaffolding domain), encoded by residues 82–101, which recognizes and binds proteins containing the sequence motif $\psi X\psi XXXX\psi$ or $\psi XXXX\psi XX\psi$, where ψ is an aromatic residue (5).

Previous studies have demonstrated that caveolin is phosphorylated on tyrosine by the oncogenic viral Src kinase (v-Src), and that caveolin is associated with normal cellular Src (c-Src) and other Src family tyrosine kinases (6, 7).

Low M_r phosphotyrosine-protein phosphatase (LMW-PTP)[1] is expressed as two molecular isoforms in mammalian species. These isoforms originated from two different mRNAs produced through an alternative splicing mechanism (8). In addition to mammals, the enzyme is expressed in a sole molecular form in several microorganism species (9–12). It belongs to the large family of protein-tyrosine phosphatases (PTPs) that are divided into four subfamilies: the tyrosine-specific phosphatases, the VH1-like dual specificity phosphatases, the cdc25 phosphatases, and the low molecular weight phosphatases. All PTPs have the active site signature sequence C*XXXXX*R, and all share the same catalytic mechanism (13). Arginine is involved in substrate binding, whereas cysteine performs the nucleophilic attack on the substrate phosphorus atom, producing a covalent enzyme-phosphate intermediate, whose hydrolysis is the limiting step of the catalytic process. An aspartic acid residue assists the nucleophilic reaction by donating a proton to the leaving group in the transition state.

Although the function of LMW-PTP in microorganisms is still debated (10, 12), several papers produced in the last few years have indicated that the enzyme participates in the tyrosine kinase receptor function (14–18). Specifically, the enzyme is involved in the down-regulation of PDGF and insulin receptors (14–16). In the case of the PDGF receptor, the overexpression of the enzyme in NIH-3T3 cells (both as wild type (wt) and

* This work was supported by Consiglio Nazionale delle Ricerche Strategic Project "Controlli Post-Trascrizionali dell'Espressione Genica" and Target Project on Biotechnology, Ministero dell' Università e della Ricerca Scientifica e Tecnologica (MURST 1997) "Meccanismi Biochimici di Controllo delle Funzioni Cellulari," MURST-CNR Biotechnology Program L.95/95, and the Italian Association for Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dipartimento di Scienze Biochimiche, Università di Firenze, Viale Morgagni 50, 50134 Firenze, Italy. Tel.: 39-055-413765; Fax: 39-055-4222725; E-mail: camici@scibio.unifi.it.

[1] The abbreviations used are: LMW, low molecular weight; PTP, phosphotyrosine-protein phosphatase; wt, wild type; dn, dominant negative; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; MES, 4-morpholineethanesulfonic acid; wtNIH-3T3, NIH-3T3 cells overexpressing wild type low molecular weight phosphotyrosine-protein phosphatase; dnNIH-3T3, NIH-3T3 cells overexpressing dominant negative low molecular weight phosphotyrosine-protein phosphatase; NIH-3T3IR, NIH-3T3 cells overexpressing insulin receptor; dnNIH-3T3IR, NIH-3T3 cells overexpressing both insulin receptor and dominant negative low molecular weight phosphotyrosine-protein phosphatase; PAGE, polyacrylamide gel electrophoresis.

as a dominant-negative (dn) mutant (C12S), a form able to bind substrates but catalytically inactive) produces opposite phenotypic effects; the wild type enzyme decreases cell growth rate, whereas the dn mutant increases it. The analysis of this action mechanism demonstrated that LMW-PTP binds and dephosphorylates the activated receptor *in vivo*. In particular, LMW-PTP is involved in pathways that regulate the transcription of the early genes *myc* and *fos* in response to growth factor stimulation.

Recent reports have also demonstrated that LMW-PTP is localized constitutively in cytosol and cytoskeleton, and that upon growth factor stimulation c-Src is able to bind and phosphorylate only the cytoskeleton-associated enzyme. As a consequence of its phosphorylation, LMW-PTP increases its activity about 20-fold, and this strongly influences both cellular adhesion and migration. The target of cytoskeleton-associated LMW-PTP is p190Rho-GAP, which is phosphorylated on tyrosine after PDGF-stimulation (19). In the case of insulin signaling, the specific involvement of the enzyme in the Src pathway has been demonstrated. Overexpression of the dn form of the enzyme also influences glucose uptake and glycogen synthesis upon insulin stimulation (16).

Finally, the involvement of LMW-PTP in the regulation of other growth factor receptors has been reported. Stein *et al*. (20) have found that LMW-PTP is involved in the ephrine-B1 receptor function; Rigacci *et al*. (18) and Rovida *et al*. (17), respectively, have shown that the fibroblast growth factor receptor and the macrophage colony-stimulating factor receptor functions are regulated by LMW-PTP.

In this paper we demonstrate that tyrosine-phosphorylated caveolin is a potential physiological substrate for LMW-PTP.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant LMW-PTP was prepared as described previously (21). Monoclonal anti-phosphotyrosine (PY-20) and polyclonal anti-caveolin (sc-894) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-phosphocaveolin (PY14) antibody was purchased from Transduction Laboratories (Lexington, KY). The sc-894 antibody is raised against a peptide corresponding to amino acids 2–21 mapping at the amino terminus of caveolin-1, while the PY-14 monoclonal antibody is raised against a tyrosine-phosphorylated peptide corresponding to amino acids 9–18 mapping at the amino terminus of caveolin-1. Rabbit polyclonal anti-LMW-PTP antibody was produced in our laboratory. Protein A-Sepharose beads was from Sigma. Soluble PTP-1B (recombinant glutathione *S*-transferase fusion protein corresponding to full-length human PTP-1B) was purchased from Upstate Biotechnology (Lake Placid, NY). Electrophoresis reagents were purchased from Bio-Rad. Enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech. All other reagents were the purest commercially available.

Protein Determination—Protein concentration was assayed by the bicinchoninic acid method (BCA kit) purchased from Sigma.

Cell Cultures and Transfections—NIH-3T3 fibroblasts, stable transfected NIH-3T3 cell lines overexpressing wild type LMW-PTP or its C12S dn mutant (prepared as described by Chiarugi *et al*. (Ref. 22)), and H-end endothelial cells (kindly provided from F. Bussolino, University of Torino, Italy) were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin). NIH-3T3IR (a cell line overexpressing insulin receptor; Ref. 23) were kindly provided by AR Saltier (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). NIH-3T3IR cells transfected with the C12S (dn) mutant gene for LMW-PTP (dnNIH-3T3IR) were prepared as described previously (16) and routinely cultured in Dulbecco's modified Eagle's medium containing with 10% fetal calf serum and 75 units/ml hygromycin, in 5% CO_2 humidified atmosphere.

Pervanadate Treatment, Preparation of Lysates, and in Vitro Dephosphorylation Experiments—Prior to cell lysis, 80–90% confluent cultures of cells (NIH-3T3 fibroblasts and H-end endothelial cells) were treated for 30 min with 0.1 mM pervanadate (20 μl of a fresh solution containing 50 mM sodium orthovanadate and 50 mM H_2O_2 was added to 10 ml of medium). Cells were collected and then lysed with 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.1 M NaCl, 1% Triton X-100, 10% glycerol, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 5 mM iodoacetate. Iodoacetate was added in order to inactivate irreversibly all intracellular PTPs (all members of PTP family contain an active site essential cysteine residue; Ref. 13). After incubation at 4 °C for 30 min, 10 mM dithiothreitol was added to inactivate any unreacted iodoacetic acid, and the insoluble material was removed by centrifugation at 102,000 × *g* for 40 min. Lysates of pervanadate-treated cells (1.5–3.5 mg of protein/ml) containing tyrosine-phosphorylated proteins were incubated at 25 °C in the absence or presence of LMW-PTP or PTP-1B (0.05–0.1 μM final concentration). Aliquots were removed at various times for analysis by SDS-PAGE and anti-phosphotyrosine immunoblotting.

Detergent-free Purification of Caveolin-rich Membrane Fractions—Low density caveolae-enriched domains were isolated as described by Song *et al*. (24). Briefly, one confluent 100-mm dish, washed twice with ice-cold phosphate-buffered saline (10 mM sodium phosphate and 0.15 M NaCl, pH 7.2), was scraped into 0.5 ml of sodium carbonate buffer (500 mM sodium carbonate, pH 11, 25 mM MES, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Cells were homogenized extensively using a Dounce homogenizer (50 strokes). The homogenate was then adjusted to 45% sucrose by the addition of 0.65 ml of 80% sucrose in 25 mM MES, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and placed at the bottom of an ultracentrifuge tube. A 5–35% discontinuous sucrose gradient was formed above the 45% layer, by adding 2.5 ml of 35% sucrose and 1.3 ml of 5% sucrose, both in 250 mM sodium carbonate, pH 11, 25 mM MES, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin. The gradient was centrifuged at 170,000 × *g* for 20 h using a Beckman SW50.1 rotor. For the analysis of the resulting gradient, 0.35-ml fractions were collected from the top to the bottom of the gradient. The insoluble pellet (fraction 15) was dissolved into 50 μl of Laemmli sample buffer. Western blot analysis was performed using both anti-caveolin and anti-LMW-PTP antibodies.

Immunoprecipitation Experiments—Confluent NIH-3T3 cells overexpressing C12S dnLMW-PTP cultured in 100-mm dishes were washed with phosphate-buffered saline, lysed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 60 mM *n*-octylglucoside, 2 mM EDTA, 1 mM orthovanadate, 100 mM NaF, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin), and insoluble material was removed by centrifugation at 10,000 × *g* for 10 min. Lysate (500 μg of protein) was incubated overnight with 1 μg of anti-caveolin antibody or 1 μg of anti-LMW-PTP antibody. After a 1-h incubation with protein A-Sepharose beads at 4 °C, the immunocomplexes were collected and washed extensively (three times) with lysis buffer. The beads were suspended in 20 μl of 2-fold concentrated Laemmli electrophoresis buffer (without 2-mercaptoethanol), separated by SDS-PAGE, and electroblotted onto polyvinylidene difluoride membranes for detection.

RESULTS

LMW-PTP Dephosphorylates Tyrosine-phosphorylated Caveolin and Few Other Tyrosine-phosphorylated Proteins Contained in Pervanadate-treated Cell Lysates—We have treated both NIH-3T3 and H-end cells with pervanadate and found that protein tyrosine phosphorylation was dramatically enhanced with respect to untreated cells, which contain virtually undetectable levels of phosphotyrosine (25). Other authors have previously reported similar findings using different cell lines (26, 27); the enhancement of tyrosine phosphorylation is due to the inhibition of all cellular PTPs by pervanadate. We have performed time-course dephosphorylation experiments by incubating lysates from pervanadate-treated H-end cells with LMW-PTP. Aliquots of the incubation mixtures were withdrawn at various times and analyzed by Western blotting with anti-phosphotyrosine antibodies (PY20). Fig. 1*A* shows the SDS-PAGE analysis, and Fig. 1*B* reports the densitometric profiles of *lanes 1–4* of *panel A*. LMW-PTP rapidly dephosphorylates a group of low molecular mass bands (<30 kDa), and at least two additional bands in the 38–55-kDa range. Other phosphotyrosine-containing proteins were also dephosphorylated, but more slowly. A similar pattern was observed using lysates from pervanadate-treated NIH-3T3 cells (data not shown).

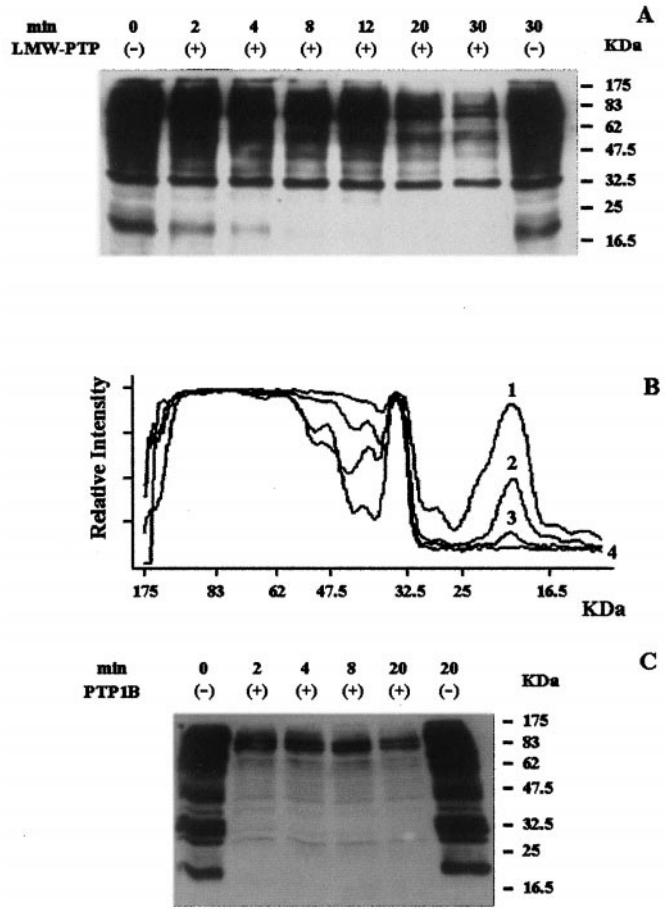

FIG. 1. **LMW-PTP dephosphorylation of some phosphotyrosine-containing proteins from pervanadate-treated H-end cell lysate.** *A*, lysates from pervanadate-treated H-end cells were incubated at 25 °C and pH 7.4 in the absence (−) or in the presence (+) of LMW-PTP. At various times (0, 2, 4, 8, 12, 20, and 30 min), aliquots containing 20 μg of protein were removed and subjected to SDS-PAGE/Western blot analysis with PY-20 anti-phosphotyrosine antibody. *B*), densitometric profiles of the immunoblots shown in *A*. *Profiles 1*, *2*, *3*, and *4* correspond to 0, 2, 4, and 8 min, respectively. *C*, an experiment similar to that shown in *A* was performed using PTP-1B instead of LMW-PTP. Similar results were obtained in at least three separate experiments.

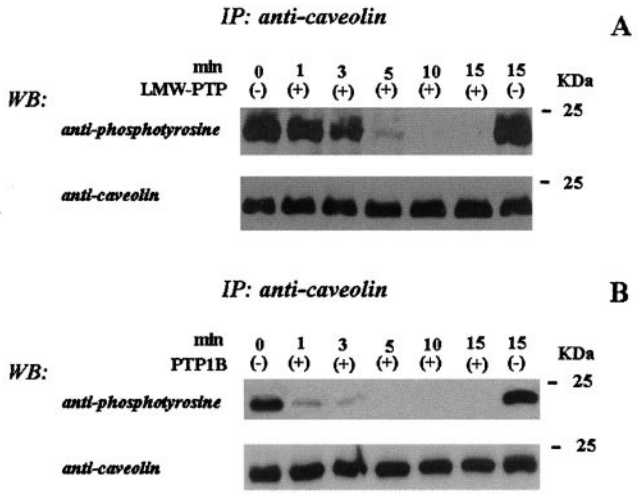

FIG. 2. **Dephosphorylation of immunoprecipitated caveolin by LMW-PTP.** Cell lysates from H-end cells were treated with sc-894 anti-caveolin antibody. The immunocomplex was collected on protein A-Sepharose, and then washed extensively with lysis buffer. Beads were suspended in 20 mM Tris-HCl buffer, pH 7.4, containing 2 mM EDTA, and incubated in the absence (−) or in the presence (+) of LMW-PTP. The incubation was performed at 25 °C. At various times (0, 1, 3, 5, 10, and 15 min), identical aliquots were removed and subjected to SDS-PAGE/Western blot analysis with PY-20 anti-phosphotyrosine and sc-894 anticaveolin antibodies. *B*, the same experiment was performed with PTP-1B instead of LMW-PTP. *IP*, immunoprecipitation; *WB*, Western blotting. Similar results were obtained in at least three separate experiments.

In order to verify the specificity of LMW-PTP in comparison with other tyrosine phosphatases, we carried out the same experiment on the cell lysate using PTP1B instead of LMW-PTP. The result is reported in Fig. 1*C*; PTP1B has the capacity to dephosphorylate a broad range of tyrosine-phosphorylated protein substrates.

Taking into account the findings of Vepa *et al*. (27), who demonstrated that caveolin is the highest phosphorylated protein with low molecular weight in pervanadate-treated endothelial cells, we performed enzymatic dephosphorylation of caveolin immunoprecipitated from the H-end cell lysate. The samples were incubated for different times with LMW-PTP. Western blot analyses performed respectively with anti-phosphotyrosine (PY-20) and anti-caveolin (sc-894) antibodies reveal that LMW-PTP rapidly dephosphorylates phosphocaveolin (Fig. 2*A*). We have also tested the activity of the phosphotyrosine-protein phosphatase PTP-1B on the anticaveolin immunoprecipitate. As expected, this enzyme dephosphorylates phosphocaveolin (Fig. 2*B*) in agreement with the finding of Fig. 1*C*, which shows that PTP-1B displays broad substrate specificity.

LMW-PTP Associates with Caveolae-enriched Membrane Domains in Vivo and Coimmunoprecipitates with Caveolin—The rationale of the following experiments, performed on NIH-3T3 fibroblasts overexpressing wild type or dominant negative LMW-PTPs, is to demonstrate both the localization of LMW-PTP in caveolae and to investigate the mechanism of its recruitment into these membrane domains. We lysed confluent cells and then separated the caveolae-enriched membrane domains from other cellular components, using the density gradient centrifugation technique described under "Experimental Procedures." Fifteen fractions were collected from the top of the gradient, and the localization of LMW-PTP and caveolin was performed by Western blotting. Fig. 3 (*A* and *B*) show that both wild type and dnLMW-PTP molecular forms are distributed in different gradient fractions; they colocalize with caveolin (fractions 4 and 5), the caveolae domain marker protein, but they are also present in fractions 12–14 (the loading zone), and in the pellet (fraction 15). In fraction 1 of the density gradient relative to the dnLMW-PTP-overexpressing cells, we have detected a positive reaction with anti-LMW-PTP antibody. The localization of both wt and dn LMW-PTPs in caveolae suggests that a completely functional active site is not required for the recruitment of the enzyme into caveolae. The LMW-PTP localization in the pellet and in the loading zone was expected, since previous papers reported that LMW-PTP is localized in both cytoplasmic and cytoskeleton-associated fractions of NIH-3T3 cells (see Ref. 19, and citations therein).

To further investigate the association of LMW-PTP with caveolin, we performed immunoprecipitation experiments with anti-LMW-PTP antibody on lysates from NIH-3T3 cells overexpressing wild type or dnLMW-PTP. Western blot analyses of the immunoprecipitate using anti-caveolin (sc-894), anti-phosphocaveolin (PY-14), and anti-LMW-PTP antibodies (Fig. 3*C*) demonstrated that cellular LMW-PTP and caveolin co-immunoprecipitate, indicating that they interact in these cells. Furthermore, the formation of LMW-PTP-caveolin complexes does not depend on caveolin phosphorylation, since we have not observed a positive reaction with the PY-14 anti-phosphocaveolin antibody. This suggests that a site different from the active

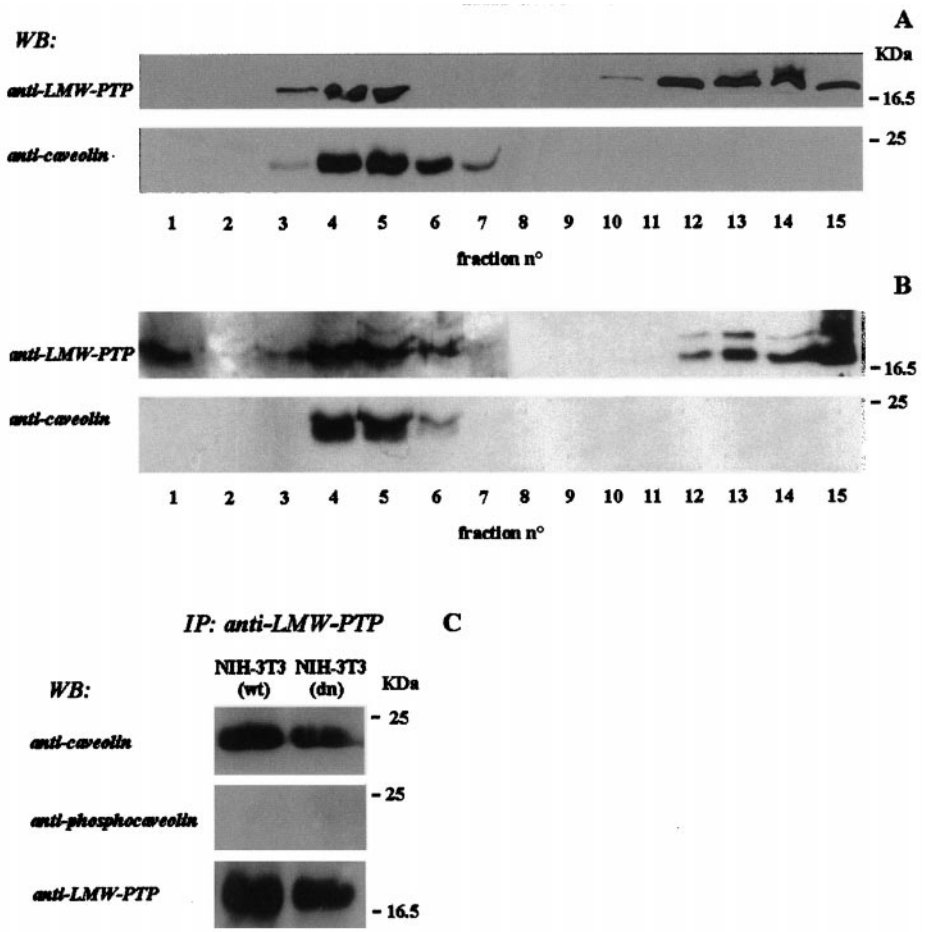

FIG. 3. **LMW-PTP associates with caveolae membranes *in vivo* and coimmunoprecipitates with caveolin.** Detergent-free sodium carbonate-based lysates were prepared from NIH-3T3 cells overexpressing wt (*A*) or dn (*B*) LMW-PTP, and fractionated by flotation in sucrose density gradient. Fractions of 0.35 ml were collected from the top to the bottom of the gradient. Aliquots (20 μl) of the fractions were subjected to SDS-PAGE/Western blot analysis with anti-LMW-PTP and sc-894 anti-caveolin antibodies. *C*, cell lysates from NIH-3T3 cells overexpressing wt or dn LMW-PTP were treated with anti-LMW-PTP antibody, and the immunoprecipitated proteins were analyzed by SDS-PAGE/Western blot analysis using anti-caveolin (sc-894), anti-phosphocaveolin (PY-14), and anti-LMW-PTP antibodies. *IP*, immunoprecipitation; *WB*, Western blotting. Similar results were obtained in at least three separate experiments.

site of LMW-PTP could be involved in the binding with caveolin.

Tyrosine-phosphorylated Caveolin Is a Cellular Substrate of LMW-PTP—The results described above suggest that tyrosine-phosphorylated caveolin is a potential substrate for LMW-PTP. In order to demonstrate this hypothesis, we have performed experiments with cells overexpressing both the insulin receptor and the dominant negative C12S LMW-PTP mutant.

Insulin-stimulated and non-stimulated cells (neoNIH-3T3IR and dnNIH-3T3IR) were lysed, and Western blots with anti-phosphotyrosine (PY-20), anti-phosphocaveolin (PY-14), and anti-caveolin (sc-894) antibodies were performed. As expected, the main effect of insulin stimulation of neo cells is an increased phosphorylation level of the 90-kDa insulin receptor β-subunit (Fig. 4*A*, *lanes 1* and *2*). Insulin stimulation of NIH-3T3IR cells overexpressing dnLMW-PTP causes a dramatic increase of tyrosine phosphorylation of the insulin receptor with respect to control cells (Fig. 4*A*, *lanes 3* and *4*). The overexpression of dnLMW-PTP is accompanied by a slight increase in the phosphorylation level of another band of approximately 60 kDa. The anti-caveolin Western blot shows that caveolin is present in the lysates (Fig. 4*C*), but the PY-20 antibody does not reveal phosphorylated caveolin (Fig. 4*A*). Nevertheless, Western blot analysis performed with the monoclonal antibody PY-14 (which recognizes Tyr^{14}-phosphorylated caveolin with high specificity) shows that upon insulin treatment caveolin phosphorylation is strongly enhanced in neo-NIH-3T3IR cells, indicating that a tyrosine kinase activated upon insulin-signaling is involved (Fig. 4*B*, *lanes 1* and *2*). The overexpression of the dominant negative LMW-PTP causes an increase in caveolin phosphorylation level both in untreated and in insulin-stimulated cells as compared with the control cells (Fig. 4*B*). Moreover, we demonstrated that, in these cells, caveolin coimmunoprecipitates with LMW-PTP (Fig. 4, *panels D–F*), and that the amount of immunoprecipitated caveolin is not dependent on its phosphorylation. In the control cells, immunoprecipitation with the anti-LMW-PTP antibody does not result in detectable LMW-PTP levels (Fig. 4*F*, *lanes 1* and *2*). Nonetheless, the samples contain significant amounts of immunoprecipitated caveolin (Fig. 4*D*). We think that this is due to the low basal level of LMW-PTP in control cells; in contrast, caveolin is revealed in the immunoprecipitate because it forms homooligomers (31), which may interact with other proteins with an unknown stoichiometry.

Taken together, the above experiments clearly demonstrate that the overexpression of the C12S LMW-PTP mutant in NIH-3T3IR cells causes the enhancement of the tyrosine phosphorylation of some specific proteins (such as insulin receptor β-subunit and caveolin) because it protects them against the action of cellular PTPs. Some authors have described these kinds of mutant as substrate traps, since, although they are catalytically inactive, they maintain the capacity to bind substrates, thus protecting them from dephosphorylation (28–30). The insulin receptor, which is immediately phosphorylated upon insulin stimulation, is an already known physiological substrate of LMW-PTP (16); our results suggest that caveolin, which is phosphorylated on tyrosine by a kinase activated upon insulin signaling, is a potential new cellular substrate for LMW-PTP.

DISCUSSION

The cellular level of protein phosphotyrosine is generally very low (25). In order to elevate cellular tyrosine phosphorylation, two approaches are currently used. First, physiological

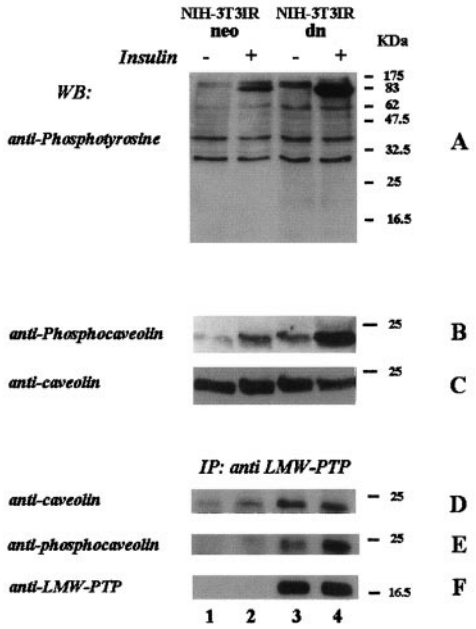

FIG. 4. **Effect of insulin stimulation on the association of endogenous tyrosine-phosphorylated caveolin with transfected dnLMW-PTP in NIH-3T3IR cells.** cDNAs encoding C12S dominant negative (*dn*) mutant of LMW-PTP or the vector alone (*neo*) were transfected in NIH-3T3IR cells. Cells were stimulated (+) or not (−) with insulin (100 nM) for 5 min, and lysates were analyzed by SDS-PAGE/Western blotting with PY-20 anti-phosphotyrosine antibody (*A*), with PY-14 anti-phosphocaveolin antibody (*B*), and with sc-894 anti-caveolin antibody (*C*). Aliquots of the lysates from the above cell lines were treated with the anti-LMW-PTP antibody, and the immunoprecipitated proteins were analyzed by SDS-PAGE/Western blotting with sc-894 anti-caveolin (*D*), PY-14 anti-phosphocaveolin (*E*), and anti-LMW-PTP (*F*) antibodies. *IP*, immunoprecipitation; *WB*, Western blotting. The results are representative of three independent experiments.

stimulation of cells with growth factors, which trigger cascades of tyrosine phosphorylation events, results in tyrosine phosphorylation of only a limited number of proteins; second, treatment of cells with pervanadate to inhibit intracellular PTPs results in the phosphorylation of a large number of proteins. We have used the latter method to obtain cell lysates and immunoprecipitates useful for revealing the tyrosine-phosphorylated proteins that are rapidly dephosphorylated by LMW-PTP *in vitro*. We performed time-course dephosphorylation experiments revealing tyrosine-phosphorylated proteins by immunoblot analysis with the PY-20 antibody. Our findings indicate that the LMW-PTP dephosphorylates some bands very rapidly, whereas it dephosphorylates other bands only after longer incubation periods.

Since Vepa *et al*. (27) reported that caveolin is one of the most tyrosine-phosphorylated proteins in pervanadate-treated endothelial cells, we performed immunoprecipitation of caveolin species using anti-caveolin antibody. The incubation of this immunoprecipitate with LMW-PTP and the successive immunoblot analyses using both anti-phosphotyrosine and anti-caveolin antibodies demonstrated that LMW-PTP very rapidly dephosphorylates phosphocaveolin *in vitro*. Furthermore, both immunoprecipitation experiments with anti-LMW-PTP or anti-caveolin antibodies and cellular fractionation experiments in wtNIH-3T3 or dnNIH-3T3 cells demonstrated that LMW-PTP is associated with caveolin in caveolae membrane enriched domains *in vivo*. Moreover, we have demonstrated that this interaction does not require caveolin phosphorylation (Fig. 3*C*); instead, it is in all probability mediated by a LMW-PTP sequence motif similar to those found in other caveolin-binding proteins. In fact, Couet *et al*. (5), using the caveolin scaffolding domain to select random peptide ligands from phage display libraries, proposed the following caveolin-binding motifs: $\psi X\psi XXXX\psi$ or $\psi XXXX\psi XX\psi$ (where ψ is an aromatic residue and *X* is any amino acid). Interestingly, in that study, nearly 10% of the selected 15-mer peptides contained only two aromatic residues. The authors also reported that, although a caveolin consensus motif including four aromatic residues ($\psi X\psi XXXX\psi XX\psi$) has been identified in several Gα subunits, Gα_q uses valine and leucine as substitutions for two of these aromatic residues. Despite this substitution, Gα_q has been shown to coimmunoprecipitate with caveolin. Thus, it seems possible that caveolin-binding requirements are broader than described previously. Recently, the caveolin-binding sequences of other caveolin-binding proteins have been described. Carman *et al*. (32) have reported that all known G-protein-coupled receptor kinases contain the conserved caveolin-binding motif (I/L)*XXXX*F*XX*F. We have observed that this caveolin-binding motif is also present in LMW-PTP (residues 77–85) (33), suggesting that caveolin interacts with this LMW-PTP region.

There is increasing evidence that caveolae play a major role in organizing signal transduction at the cell surface (3, 34). Different hormone receptors and signal transducers are localized in caveolae, and specific signaling events originate in caveolae, including the EGF-dependent activation of Raf-1 (35), interleukin-1β-stimulated production of ceramide (36), and PDGF receptor kinase cascade (37). Furthermore, caveolin-1 is directly involved in the modulation of the activity of heterotrimeric GTP-binding proteins *in vitro* (38). Thus, caveolae may be involved in the organization of these molecules, a necessary step for the integration of different sources of information during signal transduction.

Caveolin-1 exists *in vivo* as two molecular species (α and β) that differ in their NH_2-terminal sequences. These two species derive from a single gene through alternate initiation during translation; α-caveolin contains residues 1–178, whereas β-caveolin contains residues 32–178. Li *et al*. (6) have demonstrated that only α-caveolin is phosphorylated by v-Src *in vitro* and *in vivo*, and Ko *et al*. (39) detected tyrosine phosphorylation only in the β-isoform of caveolin in the Rat-1 cell line expressing a temperature-sensitive $pp60^{v\text{-}src}$ kinase. Tyrosine phosphorylation of caveolin has also been detected in normal cells, but appears to occur in a strictly regulated fashion. For example, insulin stimulates tyrosine phosphorylation of caveolin-1 only in fully differentiated 3T3-L1 adipocytes but not in fibroblasts (preadipocytes), despite the fact that both cell types express caveolin-1 and active insulin receptors (7, 40). The functional consequences of tyrosine phosphorylation of caveolin-1 are not known. However, several reports suggest some cellular implications of caveolin phosphorylation. For example, tyrosine phosphorylation of caveolin-1 has been linked to cell transformation by v-Src (41, 42). Koleske *et al*. (43) reported that cells transformed by oncogenes show reduced levels of caveolin-1 along with attenuated number of caveolae. Lee *et al*. (44) found that overexpression of caveolin-1 in oncogenically transformed cells and in breast cancer cells, where the expression of caveolin-1 is largely reduced, resulted in substantial growth inhibition and attenuation of anchorage-independent growth in soft agar. Recently, Kim *et al*. (45) reported that COOH-terminal truncation and/or overexpression of the EGF receptor in B82L fibroblasts can induce an enhanced tyrosine phosphorylation of caveolin-1 in response to EGF, suggesting that caveolin-1 plays an important role in the EGF signaling events that are mediated by aberrant forms or levels of EGF receptor. An enhanced level of wild type EGF receptor was detected in various human cancers (46, 47) and was linked with cell transformation (48–50). Furthermore, truncated EGF re-

ceptors that lack COOH-terminal autophosphorylation sites can also induce mitogenesis and cell transformation (51–54).

Among other possible effects, tyrosine phosphorylation of caveolin-1 may alter cholesterol binding to the caveolae and membrane structure (39). In fact, several studies have demonstrated that caveolin-1 is a cholesterol-binding protein (55) and that cholesterol is an important component of caveolae. Furthermore, the observed traffic of caveolin-1 between the endoplasmic reticulum and the Golgi apparatus has been implicated in the transport of cholesterol to caveolae (56). Taking into account that cholesterol level of the caveolae membrane may be involved in the regulation of mitogenesis, tyrosine phosphorylation of caveolin-1 may improve the transport of cholesterol, determining a loss of this important component in the caveolae, and thereby altering their structure/function, with consequent effects on cell growth control (45).

In the present study, using a monoclonal antibody highly specific for tyrosine-phosphorylated caveolin (PY-14) (57, 58), we have shown that the overexpression of the dn mutant of LMW-PTP (an inactive form able to bind its substrates) in NIH-3T3IR cells causes the enhancement of tyrosine-phosphorylated caveolin levels. This effect is produced by the protection exerted by dnLMW-PTP against the dephosphorylating action of endogenous LMW-PTP and other cellular PTPs on tyrosine-phosphorylated caveolin. Insulin stimulation causes a dramatic increase of the amount of phosphocaveolin level in the dnNIH-3T3IR cells with respect to neo NIH-3T3IR cells, demonstrating that phosphorylated caveolin is a cellular target for LMW-PTP.

Eukaryotic cells contain a very large number of different PTPs that probably act specifically on different cellular substrates and in different cellular sites. The identification of the physiological substrates of PTPs is a key element in understanding the biological function of this family of enzymes. Taken together, our results on the localization of LMW-PTP in caveolae, on the *in vivo* interaction between LMW-PTP and caveolin, and on the capacity of this enzyme to rapidly dephosphorylate phosphocaveolin indicate that tyrosine-phosphorylated caveolin is a relevant substrate for LMW-PTP. We suggest that this enzyme is a good candidate for the cellular regulation of phosphorylated caveolin level, with consequent implications in all the processes that are affected by caveolin phosphorylation on tyrosine.

REFERENCES

1. Rothberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y. S., Glenney, J. R., and Anderson, R. G. (1992) *Cell* **68,** 673–682
2. Anderson, R. G. W., Kamen, B. A., Rothberg, K. G., and Lacey, S. W. (1992) *Science* **255,** 410–411
3. Anderson, R. G. W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10909–10913
4. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273,** 5419–5422
5. Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272,** 6525–6533
6. Li, S., Seitz, R., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 3863–3868
7. Mastick, C. C., and Saltiel, A. R. (1997) *J. Biol. Chem.* **272,** 20706–20714
8. Dissing, J., Johnsen, A. H., and Sensabaugh, G. F. (1991) *J. Biol. Chem.* **266,** 20619–20625
9. Mondesert, O., Moreno, S., and Russell, P. (1994) *J. Biol. Chem.* **269,** 27996–27999
10. Bugert, P., and Geider, K. (1997) *FEBS Lett.* **400,** 252–256
11. Ostatin, K., Pokalsky, C., Wang, S., and Van Etten, R. L. (1995) *J. Biol. Chem.* **270,** 18491–18499
12. Grangeasse, C., Doublet, P., Vincent, C., Vaganay, E., Riberty, M., Duclos, B., and Cozzone, A. J. (1998) *J. Mol. Biol.* **278,** 339.347
13. Fauman, E. B., and Saper, M. A. (1996) *Trends Biochem. Sci.* **21,** 413–417
14. Berti, A., Rigacci, S., Raugei, G., Degl'Innocenti, D., and Ramponi, G. (1994) *FEBS Lett.* **349,** 7–12
15. Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Fiaschi, T., Camici, G., Manao, G., Romanelli, R. G., and Ramponi, G. (1998) *J. Biol. Chem.* **273,** 6776–6785
16. Chiarugi, P., Cirri, P., Marra, F., Raugei, G., Camici, G., Manao, G., and Ramponi, G. (1997) *Biochem. Biophys. Res. Commun.* **238,** 676–682
17. Rovida, E., Rigacci, S., Paccagnini, A.,. Sbarba, P. D., and Berti, A. (1998) *Biochem. Biophys. Res. Commun.* **253,** 300–304
18. Rigacci, S., Rovida, E., Bagnoli, S., Dello Sbarba, P., and Berti, A. (1999) *FEBS Lett.* **459,** 191–194
19. Chiarugi, P., Cirri, P., Taddei, L., Giannoni, E., Camici, G., Manao, G., Raugei, G., and Ramponi, G. (2000) *J. Biol. Chem.* **275,** 4640–4646
20. Stein, E., Lane, A. A., Cerretti, D. P., Schoecklmann, H. O., Schroff, A. D., Van Etten, R. L., and Daniel, T. O. (1998) *Genes Dev.* **12,** 667–678
21. Marzocchini, R., Bucciantini, M., Stefani, M., Taddei, N., Thunnissen, M. G. M. M., Nordlund, P., and Ramponi, G. (1998) *FEBS Lett.* **426,** 52–56
22. Chiarugi, P., Cirri, P., Raugei, G., Camici, G., Dolfi, F., Berti, A., and Ramponi, G. (1995) *FEBS Lett.* **372,** 49–53
23. Milarski, K. L., and Saltiel, A. R. (1994) *J. Biol. Chem.* **269,** 21239–21243
24. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 9690–9697
25. Klarlund, J. K. (1985) *Cell* **41,** 707–717
26. Garton, A. J., Flint, A. J., and Tonks, N. K. (1996) *Mol. Cell. Biol.* **16,** 6408–6418
27. Vepa, S., Scribner, W. M., and Natarajan, V. (1997) *Free Radic. Biol. Med.* **22,** 25–35
28. Flint, A. J., Tiganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1680–1685
29. Côté, J. F., Charest, A., Wagner, J., and Tremblay, M. L. (1998) *Biochemistry* **37,** 13128–13137
30. Wälchli, S., Curchod, M. L., Pescini Gobert, R., Arkinstall, S., and van Huijsduijnen, R. H. (2000) *J. Biol. Chem.* **275,** 9792–9796
31. Sargiacomo, M., Scherer, P. E., Tang, Z., Kübler, E., Song, K. S., Sanders, M. C., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9407–9411
32. Carman, C. V., Lisanti, M. P., and Benovic, J. L. (1999) *J. Biol. Chem.* **274,** 8858–8864
33. Caselli, A., Pazzagli, L., Paoli, P., Manao, G., Camici, G., Cappugi, G., and Ramponi, G. (1994) *J. Protein Chem.* **13,** 107–115
34. Anderson, R. G. W. (1998) *Annu. Rev. Biochem.* **67,** 199–225
35. Mineo, C., James, G. L., Smart, E. J., Anderson, R. G. W. (1996) *J. Biol. Chem.* **271,** 11930–11935
36. Liu, P., and Anderson, R. G. W. (1995) *J. Biol. Chem.* **270,** 27179–27185
37. Liu, P., Ying, Y., Ko, Y.-G., and Anderson, R. G. W. (1996) *J. Biol. Chem.* **271,** 10299–10303
38. Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., Lisanti, M. P. (1995) *J. Biol. Chem.* **270,** 15693–15701
39. Ko, Y.-G., Liu, P., Pathak, R. K., Craig, L. C., and Anderson, R. G. W. (1998) *J. Cell. Biochem.* **71,** 524–535
40. Mastick, C. C., Brady, M. J., and Saltiel, A. R. (1995) *J. Cell Biol.* **129,** 1523–1531
41. Glenney, J. R., Jr., and Zokas, L. (1989) *J. Cell Biol.* **108,** 2401–2408
42. Glenney, J. R., Jr. (1989) *J. Biol. Chem.* **264,** 20163–20166
43. Koleske, A. J., Baltimore, D., Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 1381–1385
44. Lee, S. W., Reimer, C. L., Oh, P., Campbell, D. B., and Schnitzer, J. E. (1998) *Oncogene* **16,** 1391–1397
45. Kim, Y.-N., Wiepz, G. J. Guadarrama, A. G., and Bertics, P. J. (2000) *J. Biol. Chem.* **275,** 7481–7491
46. Ullrich, A., and Schlessinger, J. (1990) *Cell* **61,** 203–212
47. Honegger, A. M., Szapary, D., Schmidt, A., Lyall, R., Van Obberghen, E., Dull, T. J., Ullrich, A., and Schlessinger, J. (1987) *Mol. Cell. Biol.* **7,** 4568–4571
48. Di Fiore, P. P., Pierce, J. H., Fleming, T. P., Hazan, R., Ullrich, A., King, C. R., Schlessinger, J., and Aaronson, S. A. (1987) *Cell* **51,** 1063–1070
49. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I., and Lowy, D. R. (1987) *Science* **238,** 1408–1410
50. Riedel, H., Massoglia, S., Schlessinger, J., and Ullrich, A. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1477–1481
51. Gotoh, N., Tojo, A., Muroya, K., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Yazaki, Y., and Shibuya, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 167–171
52. Sasaoka, T., Ishihara, H., Sawa, T., Ishiki, M., Morioka, H., Imamura, T., Usui, I., Takata, Y., and Kobayashi, M. (1996) *J. Biol. Chem.* **271,** 20082–20087
53. Wells, A., Welsh, J. B., Lazar, C. S., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1990) *Science* **247,** 962–964
54. Chen, W. S., Lazar, C. S., Lund, K. A., Welsh, J. B., Chang, C. P., Walton, G. M., Der, C. J., Wiley, H. S., Gill, G. N., and Rosenfeld, M. G. (1989) *Cell* **59,** 33–43
55. Murata, M., Peränen, J., Schreiner, R., Wieland, F., Kurzchalia, T. V., Simons, K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 10339–10343
56. Smart, E. J., Ying, Y., Donzell, W. C., and Anderson, R. G. W. (1996) *J. Biol. Chem.* **271,** 29427–29435
57. Lee, H., Volonté, D., Galbiati, F. Iyengar, P., Lublin, D. M., Bregman, D. B., Wilson, M. T., Campos-Gonzales, R., Bouzahzah, B., Pestell, R. G., Scherer, P. E., and Lisanti, M. P. (2000) *Mol. Endocrinol.* **14,** 1750–1775
58. Volonté D., Galbiati, F., Pestell, R. G., and Lisanti, M. P. (2001) *J. Biol. Chem.* **276,** 8094–8103