The growth factor receptor-binding protein (Grb2) has a key role in initiating the mitogen-activated protein kinase signaling cascade in major cell regulatory pathways. The binding of proteins to the SH2 domain of Grb2 has been reported to occur mainly after they are tyrosine-phosphorylated following receptor activation. Using an in vitro binding assay, immunoprecipitation, and Far Western techniques, we report that in quiescent cells a 75-kDa protein binds directly to the SH2 domain of Grb2. All of the tyrosine-phosphorylated p75 protein co-localizes with Grb2/Sos complex in the cytosolic fraction of the cell in vivo and undergoes tyrosine dephosphorylation when cells are treated with mitogenic ligands such as epidermal, platelet-derived, and fibroblast growth factors, endothelin-1, and bombesin but not tumor necrosis factor-α, interferon-γ, interleukin-6, and leukemic inhibitory factor, which are either not significantly mitogenic or not significantly mitogenic.

In vivo and in vitro phosphorylation when cells are treated with mitogenic ligands such as epidermal, platelet-derived, and fibroblast growth factors, endothelin-1, and bombesin but not tumor necrosis factor-α, interferon-γ, interleukin-6, and leukemic inhibitory factor, which are either not significantly mitogenic or not significantly mitogenic.

The SH2 domain of Grb2 binds target proteins with a phosphotyrosine consensus motif, Tyr(P)-X-Asn-X, with a preference for residues Q, Y, or V at position 1 and Y, Q, or F at position 3 relative to the phosphotyrosine (9). Proteins that contain such a motif and bind Grb2 include the EGF receptor (3), the adapter Shc (10, 11), insulin receptor substrate-1 (IRS-1) (10, 12), protein tyrosine phosphatase SHP-2 (13, 14), receptor protein tyrosine phosphatase (RPTP-α) (15, 16), and FGF receptor substrate 2 (17).

Many of the proteins that bind to the Grb2(SH2) domain are so-called “scaffold” or “docker” proteins, and it is believed that Grb2 recruits other proteins to these to form multimeric complexes that propagate various signals. Apart from its role as an adapter, Grb2 has been shown to have a role as a regulator. In unstimulated cells the binding to Grb2 was proposed to inhibit the activity of the tyrosine phosphatase RPTP-α (18). We reasoned that there may be other proteins that bind to and are possibly sequestered by Grb2 in unstimulated cells. These proteins may have important functions in signaling pathways activated by growth factors. On the other hand it may be possible, given the central role of Grb2 in cellular signaling, that its SH2 domain may be sequestered, via an association with a tyrosine-phosphorylated protein, in unstimulated cells.

We report, in unstimulated cells, a 75-kDa phosphoryrosyl protein specifically binds to the SH2 domain of Grb2 in vivo. In contrast to most proteins that bind Grb2 upon cellular activation, p75 undergoes dephosphorylation and concomitant dissociation from Grb2 upon stimulation of different cell types by various growth factors such as FGF, EGF, and PDGF.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and GST Fusion Proteins—Horseradish peroxidase-conjugated monoclonal antibodies to phosphoryrosyl and monochloral antibodies to Grb2, phosphatidylinositol 3-kinase, SHP-1, SHP-2, Raf, paxillin, cortactin, and Erk2 for Western blot analyses were purchased from Transduction Laboratories (Lexington, KY). SLP-76-tyrosine phosphatase antibody was a kind gift from Dr. A. Ullrich (Max-Planck-Institut, Munich, Germany). Secondary anti-mouse, anti-rabbit, and anti-sheep antibodies conjugated to horseradish peroxidase were from Sigma. Rabbit polyclonal anti-Grb2 antibodies for immunoprecipitation, rabbit polyclonal anti-β-gal antibodies, rabbit polyclonal anti-Sos antibodies, rabbit polyclonal c-myc antibodies, rabbit polyclonal antibodies to GST conjugated to horseradish peroxidase and agarose-conjugated GST fusion proteins, consisting of Grb2 (whole protein), Grb2 (N- and C-terminal SH3 domains), and Grb2 (SH2 domain), were from Santa Cruz Biotechnology (Santa Cruz, CA). Agarose-conjugated GST-SH2 fusion proteins for phosphatidylase C-γ, phosphatidylinositol 3-kinase, and SHP-2 were also from Santa Cruz Biotechnology. The cDNA for SH2 domain of Shc was a generous gift from Dr. T. Pawson (Mt. Sinai Hospital, Canada).
Recombinant human-EGF, mouse-TNF-α, human-LIF, mouse-IFN-α and -γ, and mouse IL-6 were obtained from Genzyme (Cambridge, MA). PIGF and bFGF were from Sigma and Boehringer Mannheim, respectively. Bombesin, vasopressin, and human porcine endothelin-1 were from Research Biochemicals International (Natick, MA). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham (Bucks, United Kingdom). Tosylphenylalanyl chloromethyl ketone (TPCK) was obtained from Boehringer Mannheim (Mannheim, FRG), dichloroacetic acid (DCIC) was from Calbiochem, and p-losyl-ar-gininine methyl ester (TAME) was from Sigma.

Cells, Cell Stimulation, and Cell Lysis—Swiss 3T3 fibroblasts (ATCC CCL92, Rockville, MD), NIH 3T3 fibroblasts (ATCC CCL10), and thymic epithelial cells (a kind gift from Dr. E. F. Potworoski, Institute for Medical Research, Haifa) were grown in 150-mm culture dishes and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, 10 mM HEPES, pH 7.4, and 100 units/ml penicillin and streptomycin. Primary human foreskin fibroblasts (HRHF) was from Whittaker Biotechnologies (Walkersville, MD) and grown in Eagle's minimum essential medium with non-essential amino acids, supplemented with 10% fetal bovine serum (Hyclone Laboratories), 2 mM glutamine, bicarbonate (0.85 g/liter), and 100 units/ml penicillin and streptomycin. The cells were lysed by 15 strokes in a Dounce homogenizer, and the supernatant was collected as the cytosolic fraction. The membrane fraction. The nuclear pellet was washed once with fractionation buffer, resuspended in lysis buffer with 1 mM EDTA, and spun at 11,000 g for 10 min at 4 °C, and the supernatant was subjected to SDS-PAGE. The gel was Western blotted, and the polyclinovylidene difluoride membrane soaked in denaturing buffer (25 mM HEPES, pH 7.9, 25 mM NaCl, 5 mM MgCl2, 0.5 mM dithiothreitol, and 0.5% Igepal CA-630) was followed by soaking in renaturation buffer (PBS containing 0.5 mM Igepal CA-630, 1 mM dithiothreitol, and 1% bovine serum albumin (w/v)) for another 5 min. The membrane was blocked in PBS containing 1% bovine serum albumin for 1 h at room temperature. After incubation with Grb2/SH2-GST fusion proteins at a concentration of 0.25 μg/ml for 1 h, the blot was washed 2 × 15 min. The bound GST fusion protein was detected by incubation with polyclonal anti-GST antibodies conjugated to horseradish peroxidase at 1:1000 dilution for 1 h. After washing for 2 × 15 min, the binding proteins were visualized using ECL kit from Amersham.

Size Fractionation Column Chromatography—Five plates (150 mm) of quiescent Swiss 3T3 cells were either left unstimulated or stimulated with 20 ng/ml EGF for 30 min. The cells were lysed by an equal volume of 2 × precipitation buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 2% Triton X-100, 2 mM EDTA, 2 mM EGTA, and 1% Nonidet P-40) was added to the cell lysate. 30 μg of GST fusion proteins containing either SH2 or SH3 domains of Grb2 conjugated to agarose beads were added to the diluted cell lysate and incubated for 2 h at 4 °C. The beads were washed three times with 1 × immunoprecipitation buffer and the bound proteins were eluted with 2 × Laemmli buffer before separation by SDS-PAGE.

Immunoprecipitation—Quiescent cells were lysed as described above and an equal volume of 2 × precipitation buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 2% Triton X-100, 2 mM EDTA, 2 mM EGTA, and 1% Nonidet P-40) was added to the cell lysate. 30 μg of the appropriate antibodies were added to the diluted cell lysate and incubated for 1 h at 4 °C, after which the secondary antibodies conjugated to agarose were added to capture the immunocomplex for 1 h at 4 °C. The beads were washed three times with 1 × immunoprecipitation buffer, and the bound proteins were eluted with 2 × Laemmli buffer before separation by SDS-PAGE.

Permevanadate Pretreatment—Quiescent cells were pretreated with permevanadate, generated by mixing 2.4 μl of 10 mM sodium vanadate and 11 μl of H2O2 (30% v/v) per 15 ml of cell medium, for 1 min and followed by stimulation with the appropriate ligands.

Subcellular Fractionation—Stimulated or unstimulated cells were washed once with cold PBS and aspirated. The cells were scraped in 1 ml of lysis buffer containing 0.5% Nonidet-P40, 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The cell lysate was spun at 11,000 g for 10 min at 4 °C, and the supernatant was used for subsequent analysis. The protein concentrations of all cell lysates were normalized after estimation of the protein content using a BCA protein assay kit from Pierce.

Binding Assays with GST Fusion Proteins—After the cells were lysed, an equal volume of 2 × precipitation buffer (20 mM Tris, pH 7.4, 300 mM NaCl, 2% Triton X-100, 2 mM EDTA, 2 mM EGTA, and 1% Nonidet P-40) was added to the cell lysate. 30 μg of GST fusion proteins containing either SH2 or SH3 domains of Grb2 conjugated to agarose beads were added to the diluted cell lysate and incubated for 2 h at 4 °C. The beads were washed three times with 1 × immunoprecipitation buffer, and the bound proteins were eluted with 2 × Laemmli buffer before separation by SDS-PAGE.

Far Western Analysis—After Grb2 immunoprecipitation, the precipitates were eluted and resolved by SDS-PAGE. The gel was Western blotted, and the polyvinylidene difluoride membrane soaked in denaturing buffer (25 mM HEPES, pH 7.9, 25 mM NaCl, 5 mM MgCl2, 0.5 mM dithiothreitol, and 0.5% Igepal CA-630) was followed by soaking the membrane in renaturation buffer (PBS containing 0.5% Igepal CA-630, 1 mM dithiothreitol, and 1% bovine serum albumin (w/v)) for another 5 min. The membrane was blocked in PBS containing 1% bovine serum albumin for 1 h at room temperature. After incubation with Grb2/SH2-GST fusion proteins at a concentration of 0.25 μg/ml for 1 h, the blot was washed 2 × 15 min. The bound GST fusion protein was detected by incubation with polyclonal anti-GST antibodies conjugated to horseradish peroxidase at 1:1000 dilution for 1 h. After washing for 2 × 15 min, the binding proteins were visualized using ECL kit from Amersham.

Results

Growth Factors Dephosphorylate p75

Growth Factors Diminish the Phosphoryrosine Level of p75, a Protein That Co-precipitates with Grb2, in Various Cell Types—The adaptor protein Grb2 has been shown to be an important link between activated receptors of various growth factors and cytokines, and the mitogen-activated protein kinase pathway (3). We and others have been characterizing tyrosine-phosphorylated proteins that bind to Grb2 in FGF-stimulated cells (20, 21). A novel 90-kDa protein was seen to bind to the SH2 domain of Grb2, and this protein, FGF receptor substrate 2 has recently been sequenced (17). During the course of studying the binding of p90 to Grb2 we observed a protein of around 75 kDa that displayed greater tyrosine phosphorylation in unstimulated cells in comparison to stimulated cells. An experiment was therefore performed to see whether this effect was seen with other growth factors. Serum-deprived Swiss 3T3 cells were treated with various concentrations of EGF, PDGF, or FGF and lysed, and immunoprecipitations of Grb2 were carried out using these lysates. The immunoprecipitates were eluted and separated by SDS-PAGE and Western blotted, and the membrane was probed with anti-phosphoryrosine antibodies. All three growth factors induced a decrease in the tyrosine phosphorylation level of p75 from Grb2 eluates, and the most prominent being EGF, in which a concentration as low as 2 ng/ml could trigger the effect (Fig. 1A). The diminution of p75 signal was very rapid, occurring within 30 s of EGF addition (data not shown), suggesting that p75 might play an early role in the signal transduction of growth factors.

There are a number of previously characterized tyrosine-phosphorylated proteins that have a molecular mass of about 75 kDa. Experiments were performed to investigate whether...
p75 corresponded to any of these known proteins. SLP-76, a tyrosine-phosphorylated protein shown to bind Grb2 in T cells, was a potential candidate for p75. However, SLP-76 is not expressed in fibroblast cells and also lacks the Grb2(SH2) consensus binding motif (22). Western blot analyses with antibodies against SLP-76 as well as other known tyrosine-phosphorylated signaling proteins of 65 to 85 kDa, including SHP-2, Raf, phosphatidylinositol 3-kinase (p85), paxillin, and cortactin were all negative (data not shown), suggesting that p75 is a novel protein.

Other factors and cytokines whose cognate receptors are known to be present in Swiss 3T3 were tested for their ability to reduce the tyrosine phosphorylation signal of the Grb2-associated p75. The neuropeptides bombesin, endothelin-1, and vasopressin, which signal through G protein-coupled receptors, induced a diminution of p75 signal in Swiss 3T3 cells (Fig. 1B, data shown only for endothelin-1). Other cytokines, such as IL-6, LIF, TNF-α, IFN-α, and IFN-γ did not affect the tyrosine phosphorylation status of p75 (data not shown).

To determine whether the diminution of p75 phosphoryrosine signal could be observed in other cell types, rat L6 myoblasts transfected with FGFR-1 (L6-R1) were treated with FGF while mouse E5 thymic epithelial cells, MRHF, and human 293 kidney epithelial cells were treated with EGF. Fig. 1C shows that tyrosine-phosphorylated p75 was also present in these diverse cell types and was similarly affected by the two growth factors (data not shown for 293 and MRHF cells).

The above data demonstrated that certain agonists induced a change in the tyrosine phosphorylation status of p75 while others did not. It is interesting that the effective ligands are those that either activate the receptor tyrosine kinases or G protein-coupled receptors but not other receptor families. These ligands also share a common feature in that they are mitogenic for Swiss 3T3 cells (23–26).

Tyrosine-phosphorylated p75 Protein Is Precipitated by the SH2 Domain of Grb2—Grb2 is an adaptor composed entirely of one SH2 domain flanked by an N and C terminus SH3 domain. An experiment was performed to show which domain precipitated p75. Swiss 3T3 cells were serum-deprived before subsequent binding studies. The cell lysates were incubated with GST fusion proteins containing the SH2 or the N- or C-terminal SH3 domain of Grb2. The associated proteins were eluted and separated by one-dimensional SDS-PAGE and Western blotted, and the membrane was probed with anti-phosphotyrosine antibodies. Fig. 2A shows that p75 can be precipitated by the SH2, but neither SH3 domain of Grb2 (lanes 3 and 4). While there were several tyrosine-phosphorylated proteins in the quiescent cells (Fig. 2A, lane 1), p75 is the major one that bound to Grb2.

To demonstrate that the Grb2(SH2)-p75 association is specific, peptide competition experiments were carried out using two phosphotyrosyl peptides as described under “Experimental Procedures.” A competitor peptide, denoted pY939, was derived from the IRS-1 sequence and contained the phosphorylated Grb2(SH2) consensus binding motif, Tyr(P)-X-Asn-X. The other peptide (pY1222), also derived from the IRS-1 sequence, did not contain the above consensus binding motif and was used as a control. Grb2(SH2)-GST fusion proteins were preincubated with either of the peptides at various concentrations before the addition of lysates from quiescent cells. The result of the peptide competition experiment is shown in Fig. 2B. There was a dose-dependent diminution of p75 phosphoryrosine signal when the concentration of the specific competitor peptide (pY939) increased from 0 to 25 μM to 50 μM (lanes 1–3). The control peptide, in contrast, did not affect p75 binding to Grb2 (lane 4).

To investigate whether p75 could bind to the SH2 domain of other signaling proteins, the respective SH2-GST fusion proteins from phospholipase C-γ, phosphatidylinositol 3-kinase, SHP-2, and Shc were employed in parallel binding studies. None of these SH2 domains was found to associate with p75 (data not shown).

Grb2 appears to be constitutively complexed to Sos via its SH3 domains (5–7). We therefore asked whether p75 was in a trimeric complex with Grb2-Sos. Swiss 3T3 cells were serum-
Growth Factors Dephosphorylate p75

Fig. 2. A, association of p75 with the SH2 domain of Grb2 in quiescent Swiss 3T3 cells. Serum-deprived cells were lysed, and the whole cell lysates were incubated with agarose-conjugated GST fusion proteins containing the SH2 (lane 2), N-SH3 (lane 3), or C-SH3 domains (lane 4) of Grb2. Approximately 30 μg of protein or 5% of total cell lysates (lane 1) were included for reference. The membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. The same membrane was stripped and probed with anti-Grb2 antibodies (lane 4), peptide competition assays of p75 binding to Grb2 in Swiss 3T3 cells. After serum deprivation, the cells were lysed, and the lysates were added to agarose-conjugated Grb2-SH2 fusion proteins after the latter had been preincubated with different concentrations of peptides for 1 h. The bound proteins were separated by 7.5% SDS-PAGE and Western blotting. The membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. Lane 1 represents Grb2-SH2 precipitation without prior incubation with peptide, while lanes 2 to 5 represent Grb2-SH2 precipitates after preincubation of Grb2-SH2 with 25 μM (lane 2) or 50 μM (lane 3) of peptide pY909, 50 μM of peptide pY1222 (lane 4), or Me2SO (lane 5), respectively. C, co-immunoprecipitation of p75 with Grb2 and Sos from quiescent Swiss 3T3 cells. Quiescent cells untreated (lane 1) or treated with EGF at 10 ng/ml for 2 min (lane 2) were lysed and subjected to immunoprecipitation using anti-Sos antibodies. The immunoprecipitates were separated by 7.5% SDS-PAGE and Western blotting, the membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. The same blot was stripped and probed for Sos and Grb2. The data shown in Fig. 3A suggest that subcellular translocation was not responsible for the diminution of p75 signal.

To assess the involvement of proteolytic degradation in the decrease of p75 signal, cells were pretreated (or not) with a mixture of cell-permeable protease inhibitors containing 20 μM TPCK, 20 μM DCM, 200 μM TAME, and 5 mM EGTA for 30 min prior to EGF stimulation. The cells were lysed, subjected to immunoprecipitation with anti-Grb2 antibodies before the bound proteins were separated by SDS-PAGE, and immunoblotted with anti-phosphotyrosine antibodies. A reduction of p75 signal was observed both without (lanes 1 and 2) and with (lanes 3 and 4) protease inhibitor pretreatment (Fig. 3B, upper panel), implying that proteolysis is not likely to be involved in the loss of phosphotyrosine signal. The effectiveness of this protease inhibition protocol was verified by its ability to abolish the previously well characterized proteolysis of Erk2 following TNF-α addition (27, 28) (Fig. 3B, lower panel).

In any cell there is a finite number of Grb2(SH2) domains available to bind to tyrosine-phosphorylated proteins bearing the appropriate binding motif. The binding affinity would differ for each protein. It is possible that in stimulated cells an excess number of phosphorylated proteins with higher affinities may displace p75 from the SH2 domain. In this case the amount of tyrosine-phosphorylated p75 would appear to decrease in Grb2 immunoprecipitates but would still be present in the post-precipitation supernatant. The displaced p75 could then be detected by binding to excess Grb2(SH2)-GST fusion proteins. Unstimulated cells or cells treated with EGF were processed for Grb2 immunoprecipitation as described in Fig. 1A. The post-precipitation supernatant from EGF-stimulated cells was then subjected to another round of precipitation using Grb2(SH2)-GST fusion proteins, and the eluted proteins were compared with the Grb2 immunoprecipitates. The result is shown in Fig. 3C. Tyrosine-phosphorylated p75 is observed only in Grb2 immunoprecipitates from unstimulated cells (lane 1) and not in immunoprecipitates (lane 2) or the SH2 precipitates (lane 3) from stimulated cells. The above data indicate that a displacement of p75 from Grb2(SH2) by other proteins that become tyrosine-phosphorylated during stimulation does not contribute to the observed decrease in the p75 signal.

Next, the possibility of dephosphorylation being responsible for the apparent loss of p75 signal was evaluated in an experi-
Growth Factors Dephosphorylate p75

All of the Tyrosine-phosphorylated p75 Is Complexed to Grb2(SH2)—In quiescent, cultured cells a number of key signaling proteins are sequestered from their potential sites of interaction using pervanadate pretreatment. Pervanadate is a cell-permeable reagent that is routinely used as a potent inhibitor of tyrosine phosphatases (29). After 1 min of pretreatment with pervanadate, cells were further treated, or not, with EGF for another 1 min. The cells were lysed, subjected to immunoprecipitation using anti-Grb2 antibodies, and proteins were visualized by ECL. Untreated cells (lanes 1 and 2) or pretreated with pervanadate (lanes 3 and 4) were fractionated into cytosol; membrane; and nucleus. Lane 1, cytosol; lane 2, membrane; and lane 3, nucleus. B, p75 tyrosine phosphorylation in Swiss 3T3 cells pretreated with a mixture of protease inhibitors prior to growth factor stimulation. Upper panel, quiescent cells were either not pretreated (lanes 1 and 2) or pretreated (lanes 3 and 4) with a mixture of protease inhibitors (5 mM EGTA, 20 μM TPCCK, 20 μM DCIC, and 200 μM TAME) for 30 min. These cells were left untreated (lanes 1 and 3) or treated with EGF at 10 ng/ml for 1 min (lanes 2 and 4) and lysed, and the lysates were subjected to immunoprecipitation with anti-Grb2 antibodies. The immunoprecipitates were resolved by 7.5% SDS-PAGE and Western blotted, the membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. Lower panel, aliquots of each fraction from unstimulated cells was incubated with bFGF at 10 ng/ml for 10 min (lanes 4–6) or pretreated with pervanadate (lanes 3 and 6) and lysed, and the lysates were subjected to immunoprecipitation with anti-Grb2 antibodies before the bound proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. The apparent inhibition of tyrosine phosphatases via pervanadate pretreatment abrogated the FGF-induced diminution of the p75 signal (Fig. 3D, lane 3, compared with lane 2). In summary, when the potential mechanisms for the observed decrease in the growth factor-stimulated p75 tyrosine phosphorylation are considered, a dephosphorylation of the protein is the most likely contributor.

FIG. 3. A, localization of p75 before and after FGF stimulation. Quiescent Swiss 3T3 cells were untreated or treated with EGF and were subsequently fractionated into various subcellular compartments. Upper panel, each fraction was incubated with agarose-conjugated full-length Grb2 GST fusion proteins, and the eluted proteins were separated by 7.5% SDS-PAGE. The gel was Western blotted, the membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. Untreated cells (lanes 1–3) and cells treated with bFGF at 10 ng/ml for 10 min (lanes 4–6) were fractionated into cytosol (lanes 1 and 4), membrane (lanes 2 and 5), and nucleus (lanes 3 and 6). Lower panel, an aliquot of each fraction from unstimulated cells was separated by 10% SDS-PAGE and immunoblotted using antibodies against selected marker proteins (SIRP, membrane; c-myc, nucleus; and Erk2, cytosol). Lane 1, cytosol; lane 2, membrane; and lane 3, nucleus. B, p75 tyrosine phosphorylation in Swiss 3T3 cells pretreated with a mixture of protease inhibitors prior to growth factor stimulation. Upper panel, quiescent cells were either not pretreated (lanes 1 and 2) or pretreated (lanes 3 and 4) with a mixture of protease inhibitors (5 mM EGTA, 20 μM TPCCK, 20 μM DCIC, and 200 μM TAME) for 30 min. These cells were left untreated (lanes 1 and 3) or treated with EGF at 10 ng/ml for 1 min (lanes 2 and 4) and lysed, and the lysates were subjected to immunoprecipitation with anti-Grb2 antibodies. The immunoprecipitates were resolved by 7.5% SDS-PAGE and Western blotted, the membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. Lower panel, quiescent Swiss 3T3 cells were either not pretreated (lanes 1–3) or pretreated (lanes 4–6) with a mixture of protease inhibitors as described above before stimulation with TNF-α at 20 ng/ml for 2 min (lanes 2 and 5) or 5 min (lanes 3 and 6). The cells were lysed, and approximately 40 μg of the total lysates from these various treatments were separated by 7.5% SDS-PAGE and Western blotted, the membrane was probed with anti-IκB antibodies, and proteins were visualized by ECL. C, assessment of the ability of other tyrosine-phosphorylated proteins to displace p75 from the SH2 domain of Grb2 in vivo. Quiescent Swiss 3T3 cells were untreated or treated with EGF (10 ng/ml) for 5 min. The cells were lysed, and the lysates were incubated with anti-Grb2 antibodies. The post-immunoprecipitation supernatant from EGF-treated cells was incubated with 30 μg of Grb2(SH2)-GST fusion proteins. The bound proteins were eluted and separated by 7.5% SDS-PAGE and Western blotted, the membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL. Lanes 1 (unstimulated) and 2 (EGF-stimulated) are Grb2 immunoprecipitates. Lane 3 is the Grb2(SH2) binding protein that remains after the Grb2 immunoprecipitation shown in lane 2. D, p75 tyrosine phosphorylation in FGF-treated cells pretreated with pervanadate. Quiescent Swiss 3T3 cells were subjected to the following treatments: no treatment (lane 1); FGF stimulation at 10 ng/ml for 10 min (lane 2); FGF stimulation after pretreatment of cells with pervanadate, generated by adding 2.4 μl of 10 mM sodium vanadate and 11 μl of H2O2 (30% v/v) per 15 ml of cell media, for 1 min (lane 3); and treatment with pervanadate alone (lane 4). After the above treatments, the cells were lysed, and the lysates were subjected to immunoprecipitation using anti-Grb2 antibodies. The immunoprecipitates were separated by 7.5% SDS-PAGE and Western blotted, the membrane was probed with anti-phosphotyrosine antibodies, and proteins were visualized by ECL.
action. For example the transcription factor NF-κB (the effector) is held in the cytosol by IκB (the regulator) and released upon ligand activation when the inhibitor protein (IκB) is phosphorylated and proteolytically degraded (27, 28). We have shown that p75 and Grb2 are complexed via tyrosine phosphorylation in quiescent cells and that this interaction is regulated by growth factors. If the controlled release of these two proteins is likely to have an important regulatory role in events, such as proliferation, it is necessary to know which protein is the regulator and which is the effector. An assessment of the relative abundance of each protein may provide an indication of which protein is likely to play which role.

Unstimulated Swiss 3T3 cells were processed for Grb2 immunoprecipitation or precipitation using Grb2(SH2)-GST fusion proteins, and the eluted proteins were compared for the level of tyrosine-phosphorylated p75. The result is shown in Fig. 5A. Lanes 2 and 3 show the amount of p75 precipitated by Grb2 immunoprecipitation or Grb2(SH2), respectively. Lanes 1 and 4 represent a second round of precipitation with Grb2(SH2-H2) to detect any unbound p75. Excess Grb2(SH2) (30 μg/aliquot) was used for the precipitation shown in lane 3. The tyrosine-phosphorylated p75 precipitated in this case would represent the maximum amount present in the aliquot of cells used in the experiment. This is verified by the absence of p75 in lane 4. In Grb2 immunoprecipitation experiments, such as shown in lane 2, the amount of p75 that is precipitable may be limited by the number of Grb2 proteins. If p75 is in excess, there should be additional p75 present in lane 1. This is, however, not the case. The amounts of tyrosine-phosphorylated p75 appear equal in lanes 2 and 3, suggesting that all p75 is bound to Grb2.

We then proceeded to determine the relative amounts of Grb-2 associated with p75. Quiescent and EGF-stimulated Swiss 3T3 cells were lysed and in turn fractionated on two Superose-12 columns in tandem as described under “Experimental Procedures.” The columns had been previously calibrated with protein standards. The buffer used to lyse the cells and to elute the proteins from the column should keep the cellular proteins in their “native” state and maintain any complexes between them. Each fraction was subjected to immunoprecipitation using anti-Grb2 antibodies. The bound proteins were separated by SDS-PAGE and immunoblotted with Grb2 antibodies. Western blot analysis for Grb2 revealed that it was present in most protein-containing fractions (data not shown).

To assess the relative amount of Grb2 protein in each fraction, densitometric readings were done on the Grb2 signals, and these are graphically represented in Fig. 5B, expressed as a percentage of the total amount of Grb2 in either stimulated or nonstimulated cell lysates. In the lysates from unstimulated cells there are two distinct peaks of Grb2, a minor peak, around 500 kDa, and a major peak, around 30 kDa. The latter peak corresponds approximately to the molecular mass of Grb2 and is likely to represent free Grb2 protein. When the cells were stimulated with EGF, it is noteworthy that the apparent free Grb2 diminishes by around 40%, and there was a concomitant increase in Grb2 associated with higher molecular mass complexes. When the protein profile from the Grb2 immunoprecipitates from unstimulated cells was assessed for tyrosine phosphorylation, p75 was not detectable. As the level of detection of p75 diminishes by around 40%, and there was a concomitant increase in Grb2 associated with higher molecular mass complexes. When the protein profile from the Grb2 immunoprecipitates from unstimulated cells was assessed for tyrosine phosphorylation, p75 was not detectable. As the level of detection of p75 decreases, Grb2 associated with higher molecular mass complexes. When the protein profile from the Grb2 immunoprecipitates from unstimulated cells was assessed for tyrosine phosphorylation, p75 was not detectable. As the level of detection of p75 decreases, Grb2 associated with higher molecular mass complexes.

From the collective data shown in Fig. 5, it can be concluded that all of tyrosine-phosphorylated p75 is bound to Grb2,
whereas only a small portion of Grb2 is bound to p75 (a maximum of 15%). In this case Grb2 is more likely to be the regulator and p75 the effector. It is possible that the roles of the two proteins could be reversed if in any cell compartment the amount of tyrosine-phosphorylated p75 exceeds the amount of Grb2.

**DISCUSSION**

The Grb2 adaptor plays a pivotal role in the Ras/Map kinase signaling cascade by recruiting Sos to the activated receptor tyrosine kinase on the plasma membrane where Ras is localized. Dominant-negative mutants of Grb2 have been shown to reverse the transformed phenotype in some cells, suggesting that Grb2 is necessary for cellular proliferation and transformation (30). A number of proteins are known to bind to the SH2 domain of Grb2 upon tyrosine phosphorylation in growth factor-stimulated cells (10–14). However, little is known about the tyrosine-phosphorylated proteins that associate with this binding module in quiescent cells. Our data for the direct association of p75 with the Grb2(SH2) domain and its dephosphorylation upon stimulation of cells by agonists presents a novel observation. It is conceivable that p75 might be part of the regulatory machinery for certain cellular functions involving Grb2, such as proliferation.

The differential effects exhibited by the various ligands on p75 dephosphorylation is intriguing. Dephosphorylation of p75 was observed with FGF, EGF, PDGF, bombesin, and endothelin-1, all of which have been reported to be mitogenic in Swiss 3T3 cells (23–26). TNF-α, LIF, IFN-α, IFN-γ, and IL-6, which are not significantly mitogenic on the same cell line, did not induce dephosphorylation of p75, even though TNF-α induced NF-κB activation and IFNs and IL-6 stimulated the tyrosine phosphorylation of the STAT proteins in these cells (data not shown). It is possible that p75 participates in the regulation of signaling pathways leading to cellular proliferation.

The dephosphorylation of p75 by the above ligands suggests the involvement of a tyrosine phosphatase(s) in the functional dissociation of the Grb2-p75 complex. This as yet unidentified tyrosine phosphatase may be a common target for both the receptor tyrosine kinases (activated by EGF, PDGF, and FGF) and the G protein-coupled receptors (activated by bombesin and endothelin-1), but it is also possible that each class of receptor can stimulate a different phosphatase, which acts on the same substrate, p75. The apparent dissociation of p75 from Grb2-Sos by the tyrosine phosphatase may free the SH2 domain and enable binding of newly tyrosine-phosphorylated proteins following receptor activation by external ligands. Evidence supporting this notion was the binding of EGFR to the Grb2-Sos complex after the apparent dissociation of p75 (Fig. 2C). If p75 were to sequester the SH2 domain of all Grb2 molecules in a particular cell compartment, inhibition of its dissociation would prevent protective binding proteins from associating with Grb2. However, our data suggest that p75 sequesters only a limited pool of Grb2, since pervanadate pre-treatment, while preventing dissociation of p75 from Grb2, did not abolish the binding of other tyrosine-phosphorylated signaling proteins, such as Shc and p90 (Fig. 3D).

The existence of different functional pools of Grb2 is in line with the observation that RPTP-α also binds the adaptor in unstimulated cells (15, 16, 18). It was proposed that this association inhibits the activity of RPTP-α, which when overexpressed has been shown to transform cells (31). This would further suggest that Grb2, in addition to its normal linker function, has a role in maintaining a key signaling protein in the “switched-off” state in quiescent cells. Parallel to this hypothesis, p75 may play an important role in signaling and Grb2 might sequester it from relevant signaling complexes. This would be likely when Grb2 is more abundant than tyrosine-phosphorylated p75. Indeed, our data seen in Fig. 5D shows this is the case. The reason for the apparent sequestering of p75 in Grb2 complexes will become clearer when p75 is purified and sequenced, and its physiological role is ascertained.

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