Identification of p125, a Component of a Group of 120-kDa Proteins That Are Phosphorylated on Tyrosine Residues in Response to Bradykinin and Bombesin Stimulation, in Anti-Ras-GTPase-activating Protein Immunoprecipitates of Swiss 3T3 Cells*

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Bradykinin (BK) and bombesin (BN) stimulate an increase in the tyrosine phosphorylation of a 120-kDa group of proteins (pps120) in Swiss 3T3 cells (Leeb-Lundberg, L. M. F., and Song X.-H. (1991) J. Biol. Chem. 266, 7746–7749). Here, we show that a component of pps120, p125, was specifically immunoprecipitated with antibodies against the p21" Ras GTPase-activating protein (GAP). The major portion of GAP in nonstimulated cells (96%) was located in the cytosol, and this distribution was not affected by exposure of cells to 1 μM BK for 1 min. A significant amount of GAP in nonstimulated cells was recovered in anti-phosphotyrosine (anti-Tyr(P)) immunoprecipitates, and the cellular distribution of this GAP essentially paralleled that of total GAP. Recovery of GAP in anti-Tyr(P) immunoprecipitates of nonstimulated cells appeared to be caused at least in part by the presence of GAP complexed to a 190-kDa tyrosine-phosphorylated protein (p190). Exposure of cells to 1 μM BK for 1 min resulted in an increase in the recovery of a portion of the cellular GAP in anti-Tyr(P) immunoprecipitates. This increase was paralleled by the appearance of a tyrosine-phosphorylated protein species of 125 kDa (p125) in anti-GAP immunoprecipitates. Tyrosine-phosphorylated p125 was present also in anti-GAP immunoprecipitates after exposure of cells to 1 μM BN. High performance gel exclusion liquid chromatography of the anti-GAP-immunoprecipitated proteins on a Protein-Pak 300SW column revealed that p125 is not GAP. Anti-GAP immunoprecipitation of p125 was prevented by prior denaturation of cell lysates in sodium dodecyl sulfate suggesting that p125 is physically associated with GAP. Chromatography of cell lysates revealed that the pps120 group of tyrosine phosphoproteins includes a 125- and a 120-kDa protein. The anti-GAP-immunoprecipitable p125 migrated identically to the 125-kDa phosphoprotein component of pps120. These observations show that the pps120 group of tyrosine phosphoproteins is composed of at least two physically distinct protein components, p125 and p120. p125 is associated in some manner with a portion of the cellular GAP after exposure of cells to BK and BN.

Swiss 3T3 fibroblast cells have served as a useful model system for the study of the signal transduction pathways stimulated by small mitogenic peptide mediators such as BK,1 BN, and vasopressin (1–8). In Swiss 3T3 cells, as well as in other cells, these peptides stimulate inositol 1,4,5-trisphosphate and diacylglycerol formation (5–8), leading to elevation of free [Ca2+], (9) and protein kinase C activity (10), respectively, and release of arachidonic acid metabolites (4, 5). The signaling pathways stimulated by these peptides are believed, at least in part, to involve G-proteins that link the cell surface peptide receptors to specific cellular phospholipases (11–16).

Several lines of evidence suggest that low molecular weight GTP-binding proteins encoded by the ras proto-oncogenes serve a role in the cell proliferative action of growth factors (17, 18). Some observations indicate that ras gene products may also be involved in cellular signaling via inositol phospholipid (19–21) and arachidonic acid (22) metabolism by small mitogenic peptides such as BK (23–26) and BN (23).

Transforming variants of the Ras proteins have an attenuated GTPase activity, and this may be important for their transforming activities (27). Recently, p21" GAP, a 120-kDa GTPase-activating protein that enhances the GTPase activity of normal variants of p21" proteins (27–30), was found to serve as a direct link between Ras proteins and membrane-associated tyrosine kinases. PDGF, EGF, Src, and Fps stimulate tyrosine phosphorylation of GAP (31–34). PDGF also promotes the formation of a membrane-signaling complex between GAP and the autophosphorylated PDGF receptor (32–34) that depends on the presence of the SH2 and SH3 regions in GAP (35). This complex may be responsible for the ability of PDGF to stimulate formation of GTP-complexed p21" (36).

We reported recently that BK and BN stimulate an increase in tyrosine phosphorylation of a 120-kDa group of proteins, designated pps120, in Swiss 3T3 cells (37). Here, we show that a component of pps120, p125, was recovered in the anti-GAP immunoprecipitates of these cells.

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EXPERIMENTAL PROCEDURES

Materials—Polyclonal anti-human rGAP (anti-GAP) antibody was prepared by Dr. R. Halenbeck (38) and was the generous gift of Cetus Corp. Emergency, CA. Mixed monoclonal anti-bovine phosphoprotein Cγ-1 antibody was the generous gift of Dr. Sue Goo Rhee (NIH) and was obtained from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-phosphotyrosine (anti-Tyr(P)) antibody (py20) was from ICN (Costa Mesa, CA), and agaorce-agarose monoclonal anti-Tyr(P) antibody was from Oncogene Science (Uniondale, NY). All other biochemicals were obtained as described previously (37) and always of the highest grades available.

Cell Growth—Murine Swiss 3T3 cells were obtained from American Type Culture Collection and grown essentially as described previously (37). The growth medium was replaced with Dulbecco’s modified Eagle’s medium (DMEM) for 1 h before the experiment. At 15 min before the experiment, the medium was replaced with 1 ml of fresh Dulbecco’s modified Eagle’s medium. Cells were stimulated with 1 μM BK or 1 μM BN for 1 min as indicated in the figure legends and then washed twice with ice-cold phosphate-buffered saline containing 1 mM sodium orthovanadate.

Immunoprecipitation—In general, cells grown on 10-cm diameter dishes were lysed and solubilized in 1 ml of ice-cold immunoprecipitation buffer (1% Nonidet P-40 in 20 mM Tris-Cl, pH 8.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, including the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), 1 mM NaF, 50 mM HEPES, 50 mM NaCl, 0.5 mM dithiothreitol, and 1 mM sodium orthovanadate). Cells contain a significant amount of tyrosine-phosphorylated proteins, and immunoprecipitates of lysates of nonstimulated cells contain a significant amount of tyrosine-phosphorylated GAP. For immunoprecipitation of tyrosine phosphoproteins, soluble extracts were incubated with 2% SDS in a Dounce homogenizer (40 strokes). After heating the precipitate at 100°C for 3 min, cooled to 4°C, forced through a 25-gauge needle, and filtered (0.45 μm). Extracts (0.4 ml) were chromatographed on a protein-A-Sepharose slurry in immunoprecipitation buffer) and continued incubation for 1 h. The beads from each of the immunoprecipitations were collected by centrifugation at 13,000 × g for 1 min and washed three times with ice-cold immunoprecipitation buffer and one time with 10 mM Tris-HCl, pH 7.4. For SDS-polyacrylamide gel electrophoresis of the immunoprecipitated proteins, proteins were eluted from the beads by heating the sample at 100°C for 5 min in SDS-polyacrylamide gel electrophoresis buffer.

Disruption of protein-protein complexes before immunoprecipitation was done essentially as previously described (39). In short, cells were lysed in 10 mM sodium phosphate, pH 7.0, 0.5% SDS, 2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were heated at 100°C for 3 min, cooled to 4°C, forced through a 25-gauge needle, and diluted with the immunoprecipitation buffer before immunoprecipitation.

Immunoblotting—Proteins (~100 μg) were separated by SDS-polyacrylamide gel electrophoresis on 7% gels and immunoblotted essentially as previously described (37).

Gel Exclusion Chromatography—For chromatography of cell lysates, cells grown on 10-cm diameter dishes were collected by gently scraping into 1 ml of ice-cold phosphate-buffered saline containing 0.1 mM sodium orthovanadate and centrifuged at 13,000 × g for 15 s. Cells were then resuspended in 0.3 ml of chromatography buffer (1% Nonidet P-40 in 50 mM HEPES, pH 7.0, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The suspension was stored on ice for 15 min. The suspension was centrifuged at 13,000 × g for 20 min and then filtered (0.45 μm). For chromatography of immunoprecipitated proteins, proteins were dissociated from the antibodies bound to the protein A-Sepharose beads by heating the precipitate at 100°C for 5 min in 2% SDS, 0.1 M Tris-HCl, pH 7.0, 10 mM NaCl, 0.5 mM EDTA, and 0.5 ml/min using a Waters HPLC system. The fractions (0.25 ml) were visualized directly.

Cell Fractionation—Cells grown on 10-cm diameter dishes were collected by gently scraping into 1 ml of ice-cold hypotonic buffer (20 mM HEPES, pH 7.4, 5 mM EDTA, 1 mM MgCl₂, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate) including protease inhibitors and lysed in a Dounce homogenizer (40 strokes). After centrifugation at 1,000 × g for 5 min, the supernatant was centrifuged at 100,000 × g for 45 min. The cytosolic supernatant (S-100) was removed and the pellet resuspended in fresh buffer and refrozen. The pellet was then solubilized for 30 min with ice-cold 1% Triton X-100 in 50 mM HEPES, pH 7.4, 50 mM NaCl, 0.5 mM EDTA, 1 mM sodium p-nitrophenyl phosphate, and 1 mM sodium orthovanadate including protease inhibitors. The soluble extract of the pellet (P-100 fraction) and the S-100 fraction were then concentrated to ~1 ml using an Amicon ultrafiltration membrane cone by centrifugation at 600 × g. Sufficient Triton X-100 was then added to the S-100 fraction to make it 1%.

Other Assays—Proteins were determined using Bradford’s method (40). Densitometry was performed on selected autoradiograms using a computer–coupled video system, and the data were analyzed by the NIH image program.

RESULTS AND DISCUSSION

Swiss 3T3 cell lysates were immunoblotted with anti-GAP antibodies to ascertain the presence of GAP. Fig. 1A, lane 1, shows that GAP was not detected in nonstimulated lysates. Stimulation of cells with 1 μM BK for 1 min did not change the total amount of GAP in the cells (Fig. 1A, lane 2). As shown in Fig. 1C, the major portion of the GAP (96%) was located in the cytosolic (S-100) fraction (lanes 1 and 3). No apparent change in the cellular distribution of total GAP was observed when cells were stimulated with 1 μM BK (Fig. 1C, lanes 2 and 4).

GAP is phosphorylated on tyrosine residues within minutes of exposure of certain fibroblast cell lines to growth factors such as PDGF and EGF (31–34). The presence of phosphorylated GAP in Swiss 3T3 cells was first evaluated by anti-GAP immunoblotting of anti-Tyr(P) immunoprecipitates. Anti-Tyr(P) antibodies immunoprecipitated a significant amount of GAP from lysates of nonstimulated cells (Fig. 1B, lane 1). The distribution of the anti-Tyr(P) immunoprecipitable GAP between the cytosol (S-100) and membrane (P-100) effect is obscured. As shown in Fig. 1D, lanes 1 and 3) essentially followed that of total GAP (Fig. 1C, lanes 1 and 3). These results show that Swiss 3T3 cells contain a significant amount of tyrosine-phosphorylated GAP and/or GAP that is physically associated with a tyrosine-phosphorylated protein(s) in the absence of growth factor stimulation. These results contrast with those obtained using NIH3T3 cells, which showed that anti-Tyr(P)-immunoprecipitable GAP was recovered only after growth factor stimulation (32).

No BK-promoted change in the amount of GAP recovered in the anti-Tyr(P) immunoprecipitates was apparent when the whole cell lysate (Fig. 1B, lanes 1 and 2) or the cytosol (Fig. 1D, lanes 1 and 2) was immunoprecipitated. On the other hand, a significant increase in the amount of GAP in the anti-Tyr(P) immunoprecipitates after BK stimulation was observed when the membrane fraction was used (Fig. 1D, lanes 3 and 4). These results suggest that BK is able to stimulate an increase in the phosphotyrosine content of either GAP or a protein that is associated with GAP. The absence of an effect of BK on the cytosolic GAP can be interpreted in at least two ways. One interpretation is that this pool of GAP is modulated only a small fraction of the cytosolic GAP. Consequently, because of the high phosphotyrosine content of the cytosolic GAP under control conditions, the BK-specific effect is obscured.

To determine if BK stimulated tyrosine phosphorylation of GAP and/or tyrosine phosphorylation of a GAP-associated protein, anti-GAP immunoprecipitates of lysates of nonstimulated and BK-stimulated cells were immunoblotted with anti-Tyr(P) antibodies. Anti-GAP immunoprecipitates of nonstimulated and stimulated cells contained a major 190-
Fig. 1. Identification of total GAP and anti-Tyr(P)-immunoprecipitable GAP in BK-stimulated Swiss 3T3 cells. A, cells were incubated in the absence (lane 1) and presence of 1 μM BK (lane 2) for 1 min. Whole cell lysates were then immunoblotted with anti-GAP antibody. B, cells were incubated in the absence (lane 1) and presence of 1 μM BK (lane 2) for 1 min. Whole cell lysates were then immunoprecipitated with agarose-coupled anti-Tyr(P) antibody and immunoblotted with anti-GAP antibody. C, cells were incubated in the absence (lanes 1 and 3) and presence of 1 μM BK (lanes 2 and 4) for 1 min. The cells were then fractionated into the cytosolic fraction (S-100) (lanes 1 and 2) and membrane fraction (P-100) (lanes 3 and 4) as described under "Experimental Procedures." Protein concentrations (mg/ml) in the S-100 and P-100 fractions, respectively, were 2.1 and 1.8 (no additions) and 2.2 and 1.6 (plus BK). Aliquots (80 μl) of the fractions were immunoblotted with GAP antibody. D, aliquots (1 ml) of the fractions described under C were first immunoprecipitated with agarose-coupled anti-Tyr(P) antibody and then immunoblotted with GAP antibody. The arrows indicate the position of GAP. Molecular weight standards are shown. K, kilodaltons.

Fig. 2. Identification of p125 in anti-GAP immunoprecipitates of BK- and BN-stimulated Swiss 3T3 cells. A, cells were incubated in the absence (lane 1) and presence of 1 μM BK (lane 2) for 1 min. Whole cell lysates were then directly immunoblotted with anti-Tyr(P) antibody. The arrow indicates pps120. B, cells were incubated in the absence (lane 1) and presence of 1 μM BK (lane 2) or 1 μM BN (lane 3) for 1 min. Whole cell lysates were immunoprecipitated with anti-GAP antibody and then immunoblotted with anti-Tyr(P) antibody. The upper arrow indicates p190, and the lower arrow indicates p125. C, cells were incubated in the presence of 1 μM BK for 1 min. The whole cell lysates were immunoprecipitated with nonimmune rabbit immunoglobulin (lane 1) and anti-GAP antibody (lane 2) at approximately 40 μg of immunoglobulin protein per immunoprecipitation and then immunoblotted with anti-Tyr(P) antibody. The upper arrow indicates p190, and the lower arrow indicates p125. D, cells were incubated in the presence of 1 μM BK for 1 min. The whole cell lysates were immunoprecipitated with anti-GAP antibody, and the immunoprecipitate (immunoprcpt.) (lane 1) and supernatant (sup.) (lane 2) were immunoblotted with anti-Tyr(P) antibody. The left side arrow indicates p125, and the right side arrow indicates p120. E, the precipitate (immunoprcpt.) (lane 1) and the supernatant (sup.) (lane 2) in D were immunoblotted with anti-GAP antibody. The arrow indicates the position of GAP.
kDa tyrosine phosphoprotein (p190) (Fig. 2B, lanes 1–3, upper arrow). A protein of similar molecular weight has been shown to be phosphorylated on tyrosine residues in v-src-transformed cells and after exposure of various fibroblast cell lines to growth factors (31–34, 39). This protein forms a complex with GAP, and the formation of the complex is dependent on phosphorylation of the protein (39). The presence of tyrosine-phosphorylated p190 in anti-GAP immunoprecipitates of Swiss 3T3 cells is supportive of a complex of p190 and GAP in these cells. Neither BK nor BN altered the phosphotyrosine content of p190.

BK stimulates an increase in tyrosine phosphorylation of a 120-kDa group of proteins (pps120) in Swiss 3T3 cells (37) (Fig. 2A, lanes 1 and 2, arrow). This response is seen also after stimulation with BN (37). After BK or BN stimulation, a broad band centered at $M_r$ ≈ 125 kDa (p125) was clearly present on anti-Tyr(P) immunoblots of anti-GAP immunoprecipitates (Fig. 2B, lanes 1–3, lower arrow). Neither p125 nor p190 was recovered in nonimmune rabbit immunoglobulin immunoprecipitates (Fig. 2C, lane 1) or in anti-bovine phospholipase C$_\gamma$-1 immunoprecipitates (data not shown). Thus, immunoprecipitation of p125 and p190 was apparently specific for anti-GAP antibodies (Fig. 2C, lane 2, arrows). Anti-Tyr(P) blots of the supernatant from the anti-GAP immunoprecipitation of BK-stimulated cells showed a band at $M_r$ ≈ 120 kDa (p120) (Fig. 2D, lane 2, right side arrow). The BK-sensitive p120 phosphoprotein appeared sharper on the immunoblots and migrated with an $M_r$ slightly smaller than the BK-sensitive p125 phosphoprotein that was immunoprecipitated by the anti-GAP antibodies (Fig. 2D, lane 1, left side arrow). These results suggest that BK stimulates tyrosine phosphorylation of at least two proteins of very similar molecular weights in these cells. Furthermore, these two proteins may be distinguished by immunoprecipitation with anti-GAP antibodies. Fig. 2E, lanes 1 and 2, shows that virtually all GAP present in the cells was immunoprecipitated by the anti-GAP antibodies. These results suggest that the BK-sensitive p120 that was identified in the supernatant from the anti-GAP immunoprecipitation (Fig. 2D, lane 2, right side arrow) is not GAP.

Because of the apparently specific immunoprecipitation of p125 by anti-GAP antibodies, we needed to evaluate the relationship between p125 and GAP. To do so, we performed HPLC gel exclusion chromatography of proteins in anti-GAP immunoprecipitates of cell lysates from BK-stimulated cells using a Protein-Pak 300SW column, and the fractions from these runs were then immunoblotted with anti-Tyr(P) or anti-GAP antibodies. Immunoprecipitated proteins were dissociated from the antibody-protein A-agarose complex with a low concentration of SDS (2%). Fig. 3A shows an anti-Tyr(P) immunoblot of the fractions from a chromatographic run of the anti-GAP immunoprecipitated proteins from BK-stimulated cells. Fig. 3B shows an anti-GAP immunoblot of the fractions from the same run. The BK-sensitive p125 was the major tyrosine phosphoprotein in the fractions assayed (Fig. 3A, top arrow indicates the fraction with the peak p125 activity). When comparing the anti-Tyr(P) immunoblot in Fig. 3A with the anti-GAP immunoblot in Fig. 3B, it is clear that p125 behaved as a protein with a higher apparent molecular weight than GAP. These results demonstrate that the BK-sensitive p125 identified in anti-GAP immunoprecipitates is not GAP. The anti-GAP immunoprecipitate also contained a minor tyrosine phosphoprotein of $M_r$ ≈ 120 kDa. This phosphoprotein co-migrated with GAP and may be tyrosine-phosphorylated GAP (compare Fig. 3, A and B). The tyrosine phosphorylation of this protein was not increased appreciably by BK stimulation (data not shown).

HPLC gel exclusion chromatography was used also to probe the relationship between p125 and the proteins constituting pps120. Fig. 3C shows an anti-Tyr(P) immunoblot of fractions from a chromatographic run of the cell lysates of BK-stimulated cells. Fig. 3D (top autoradiogram) shows an anti-GAP immunoblot of the same fractions. Gel exclusion chromatography fragmented pps120 into primarily two phosphoprotein

**Fig. 3. Fractionation of GAP and BK-sensitive p125 and p120 by gel exclusion chromatography.** Cells were incubated in the absence (–BK) or presence of 1 μM BK (+BK) for 1 min. A. Whole cell lysates were immunoprecipitated with anti-GAP antibodies. The eluted proteins were then subjected to HPLC gel exclusion chromatography on a Protein-Pak 300SW column, and fractions (0.25 ml) were immunoblotted with anti-Tyr(P) antibody. The top arrow indicates a fraction with peak p125 activity. B. Fractions in A were immunoblotted with anti-GAP antibody. The left side arrow indicates position of GAP. C. Whole cell lysates were subjected to HPLC gel exclusion chromatography on a Protein-Pak 300SW column, and fractions (0.25 ml) were immunoblotted with anti-Tyr(P) antibody. The top right arrow indicates fraction with peak activity of a lower molecular weight component of pps120, and the top left arrow indicates fraction with peak activity of a higher molecular weight component of pps120. D. Top autoradiogram, fractions in C were immunoblotted with anti-GAP antibody; bottom autoradiogram, anti-GAP immunoblot of a chromatographic run of lysates of nonstimulated cells. The left side arrows indicate the position of GAP. The arrow below each autoradiogram indicates the direction of decreasing molecular weight (MW) in the gel exclusion chromatography run.
components (Fig. 3C, top arrows indicate the fractions with the peak activities). A major 120-kDa phosphoprotein (Fig. 3C, right arrow) migrated virtually identically to GAP (compare Fig. 3, C and D). A minor 125-kDa phosphoprotein (Fig. 3C, left arrow) migrated as a protein with a higher apparent molecular weight than GAP (compare Fig. 3, C and D). Interestingly, the 125-kDa phosphoprotein component of pps120 migrated identically to p125, identified in anti-GAP immunoprecipitates (compare Fig. 3, A, arrow and C, left arrow). Thus, we believe strongly that p125 is a component of the pps120 group of tyrosine phosphoproteins. The major 120-kDa phosphoprotein component of pps120 identified in Fig. 3C (right arrow) probably represents the p120 phosphoprotein identified in Fig. 2D (lane 2, right arrow), which remained in the supernatant from anti-GAP immunoprecipitations. These results provide further evidence that pps120 is composed of structurally different tyrosine phosphoproteins. Fig. 3D also shows that BK did not significantly change the behavior of GAP on the gel exclusion column.

To investigate if the BK-sensitive p125 was immunoprecipitated by anti-GAP antibodies as a consequence of a physical interaction with GAP or as a consequence of interacting directly with the antibodies, lysates of BK-stimulated cells were heated for 3 min at 100 °C in 0.5% SDS before dilution and immunoprecipitation with anti-GAP antibodies and subsequent immunoblotting with anti-Tyr(P) antibodies. Fig. 4A (lanes 1 and 2, right side, lower arrow) shows that denaturation decreased immunoprecipitation of the BK-sensitive p125. A tyrosine phosphoprotein of a Mr ~ 120 kDa, which appeared sharper on the immunoblot than p125, was still present in the anti-GAP immunoprecipitates after denaturation (Fig. 4A, lane 1, left side arrow). Fig. 4B, lanes 1 and 2, shows that immunoprecipitation of GAP was unaltered by denaturation. Thus, the tyrosine phosphoprotein present in the anti-GAP immunoprecipitate after denaturation may be tyrosine-phosphorylated GAP (Fig. 4A, lane 1, left side arrow). Fig. 4A (lanes 1 and 2, right side upper arrow) shows that denaturation also decreased immunoprecipitation of p190. These results suggest that the BK-sensitive p125 that was present in anti-GAP immunoprecipitates, in analogy with p190, was not immunoprecipitated directly by anti-GAP antibodies but rather as a complex with GAP.

Anti-Tyr(P) immunoblots of anti-GAP immunoprecipitates of different cellular fractions indicated that most of the GAP-p190 complexes in Swiss 3T3 cells were cytosolic (S-100) (Fig. 5, lanes 3 and 4, right side upper arrow), and this result agrees with that obtained using v-src-transformed Rat-2 cells (39). The BK-sensitive p125 also seemed to be primarily cytosolic (S-100), even though a small amount of this protein appeared to be present also in the membrane (P-100) (Fig. 5, lanes 3 and 4, right side lower arrow). The BK-sensitive p120 that remained in the supernatant from anti-GAP immunoprecipitations was also located primarily in the cytosol (S-100) (Fig. 5, lanes 1 and 2, left side arrow). Anti-Tyr(P) immunoblots of the cytosolic (S-100) and membrane (P-100) fractions of BK-stimulated cells that had not been immunoprecipitated with anti-GAP antibody indicated that pps120 displayed a cellular distribution similar to that of p125 and p120 (data not shown).

In summary, these observations indicate that BK- and BN-sensitive p125 is immunoprecipitated specifically with anti-GAP antibodies after BK or BN stimulation. Several results suggest that p125 exists in a complex with GAP. These results

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**Fig. 4. Evidence for a GAP complex with p125 in BK-stimulated Swiss 3T3 cells.** Cells were incubated in the presence of 1 μM BK for 1 min. A, the cells were lysed in buffer containing 0.5% SDS (lane 1) or in buffer containing 1% Nonidet P-40 (lane 2). The lysates containing SDS were denatured by heating at 100 °C for 3 min before dilution and immunoprecipitation. Denatured (D) (lane 1) and nondenatured (N) (lane 2) lysates were immunoprecipitated with anti-GAP antibody and immunoblotted with anti-Tyr(P) antibody. Left side arrow indicates a tyrosine phosphoprotein, which was immunoprecipitated after denaturation. Right side lower arrow indicates p190 and right side lower arrow indicates p125. B, the denatured (D) (lane 1) and nondenatured (N) (lane 2) samples in A were immunoblotted with anti-GAP antibody. The arrow indicates the position of GAP.

**Fig. 5. Cellular distribution of p125, p120, and p190 in Swiss 3T3 cells.** Cells were incubated in the presence of 1 μM BK for 1 min. The cells were then fractionated into the cytosolic fraction (S-100) (lanes 1 and 3) and membrane fraction (P-100) (lanes 2 and 4). The fractions were immunoprecipitated with anti-GAP antibody, and the immunoprecipitates (immunoprcpt.) (lanes 3 and 4) and supernatants (sup.) (lanes 1 and 2) were immunoblotted with anti-Tyr(P) antibody. The left side arrow indicates p120 in the supernatant, the right side upper arrow indicates p190 in the immunoprecipitate, and the right side lower arrow indicates p125 in the immunoprecipitate.
GAP in platelets after thrombin stimulation, and these kinases were proposed to mediate thrombin-promoted tyrosine phosphorylation of GAP in this system (42). The receptors for thrombin, BK, and BN are members of the seven-transmembrane, G-protein-coupled receptor superfamily and lack tyrosine kinase homology regions (43-45). Thus, Src family protein tyrosine kinases are possible candidates for mediating the effect of BK and BN on tyrosine phosphorylation.

Unlike several other fibroblast cell lines, nonstimulated Swiss 3T3 cells contain a significant amount of GAP in anti-Tyr(P) immunoprecipitates (31-34, 39). These results indicate that nonstimulated Swiss 3T3 cells contain a significant amount of tyrosine-phosphorylated GAP and/or GAP complexed to tyrosine-phosphorylated proteins. The exact reason for this result is unknown, but it suggests that one or more tyrosine kinases are constitutively active in these cells. Anti-Tyr(P)-immunoprecipitable GAP and anti-GAP-immunoprecipitable p190 displayed the same cellular distribution (primarily cytosolic). Thus, p190 is probably one protein that is complexed to GAP in nonstimulated Swiss 3T3 cells and contributes to the presence of GAP in anti-Tyr(P) immunoprecipitates.

In all, our results, together with those of others (43), suggest that some mitogenic agonists that stimulate cells through G-protein-coupled receptors can influence the p21^{GTP}/Ras-GAP system. In terms of BK- and BN-stimulated Swiss 3T3 fibroblasts, our results suggest that the mechanism involves tyrosine phosphorylation of the putative GAP-complexed protein p125. In terms of thrombin-treated platelets, the mechanism underlying this regulation appears to be a direct tyrosine phosphorylation of GAP (42). The functional consequence of either of these events is still unknown, but they may result in modulation of p21^{GTP} activity.

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Ras-GAP and p125