Tyrosine Phosphorylation of Ras GTPase-activating Protein Stabilizes Its Association with p62 at Membranes of v-Src Transformed Cells*

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Ras GTPase-activating protein (GAP) regulates the activity of Ras proteins, which have key roles in signal transduction pathways downstream of oncogenic and receptor tyrosine kinases. Previous studies indicated that Tyr-457 of bovine GAP (Tyr-460 of human GAP) is the major site of phosphorylation by viral Src (v-Src) kinase and epidermal growth factor receptor. The finding that Tyr-457 in GAP is located immediately adjacent to Src homology 2 (SH2) and 3 (SH3) domains led us to investigate the possibility that this specific phosphorylation regulates protein-protein interactions involving GAP. For this purpose, we constructed a full-length GAP mutant containing a substitution of Phe-457 in place of Tyr-457, while the amino-terminal portion containing Tyr-457 was weakly phosphorylated on tyrosine and, as expected, lacked the phosphopeptide containing Tyr-457. Analysis of GAP-associated proteins in anti-KT3 immunoprecipitates showed that GAP stably associated with two major phosphoproteins, p62 and p190, which have been previously described. Significantly, association of p62 with GAP was reduced approximately 3-fold compared with wild-type GAP. Subcellular fractionation experiments further demonstrated that Tyr-457 phosphorylation of GAP stabilized its association with p62 at cell membranes. Based on these findings, we propose that one role of tyrosine phosphorylation in GAP is to enhance its association with p62 at membranes, which in turn may contribute to regulation of Ras signal transduction pathways.

Ras protein has a central role in regulation of normal cell proliferation and in oncogenesis (1). Ras functions like a GTP-binding regulatory protein (G-protein) with a GTP/GDP cycle that is controlled by other proteins (1-4). Normal cellular Ras is biologically and biochemically active only when bound to GTP, and oncogenic activation of Ras is induced by mutations that either inhibit GTP hydrolysis or facilitate GTP/GDP exchange (2-5). The current hypothesis regarding signalling pathways involving Ras is that the Ras-GTP complex binds to effector molecules and transmits signals before GTP hydrolysis returns Ras to the inactive GDP complex (2-4, 6). Much evidence indicates that Ras acts downstream of tyrosine kinases, including viral Src (v-Src),1 in mitogenic signalling pathways and that tyrosine kinases can activate Ras function (6-10). However, the mechanism by which this Ras activation by upstream tyrosine kinases occurs is not completely understood. Ras GTPase-activating protein (GAP) was discovered by its ability to stimulate the intrinsic GTPase activity of Ras by over 100-fold (11). GAP interacts with cellular Ras as well as the active GTP-bound form and inactivates Ras by stimulating its GTPase activity (2, 3, 11-13). This negative regulatory function of GAP is consistent with the finding that overexpression of human GAP blocks oncogenic transformation by cellular Ras (14). In addition to a regulatory function, GAP may be required for Ras effector function, possibly as one of the downstream target molecules of Ras (2, 4, 15). Recent studies also point to the important regulatory role of guanine-nucleotide releasing proteins, which convert the inactive Ras-GDP complex to the active GTP complex by stimulating nucleotide exchange (16-21). Mitogenic signal transduction through Ras, therefore, involves stimulation of nucleotide exchange factors in addition to GAP function.

Ras GTPase-enhancing activity of GAP resides in the carboxy-terminal region (22), while the amino-terminal portion contains Src homology 2 (SH2) and 3 (SH3) regions (13). SH2 regions have been shown to bind phosphorylated tyrosine residues in proteins and are implicated in the interactions of tyrosine kinases with their targets (23-25). GAP is phosphorylated by a variety of oncogenic tyrosine kinases, including v-Src, as well as by the ligand-stimulated receptor tyrosine kinases (26, 27). GAP also forms complexes with the platelet-derived growth factor receptor and Src kinase, interactions which can be mimicked in vitro by using either baculoviral expressed GAP or bacterially expressed GAP SH2 domains (24, 28-31). These findings suggest that GAP might serve as a link between tyrosine kinases and Ras in mitogenic signalling pathways.

GAP forms complexes with two additional phosphoproteins, p62 and p190, in cells expressing activated tyrosine kinases, including v-Src (27, 32). The isolated GAP SH2 domains expressed in bacteria can form stable complexes in vitro with p62 (25), suggesting that GAP complex formation with p62 is a specific SH2-mediated interaction. Activation of tyrosine kinases may induce the formation of signalling complexes involving GAP, p62, and p190, which could in turn regulate signal transduction through Ras. Recently, we and others have shown that Tyr-457 of bovine GAP (corresponding to Tyr-460 of human GAP) is the major tyrosine residue phosphorylated by both activated Src kinase and the epidermal growth factor receptor kinase (33, 34).

1 The abbreviations used are: v-Src, viral Src; GAP, GTPase-activating protein.

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located immediately COOH-terminal to one of two SH2 regions in GAP, this specific phosphorylation might regulate protein-protein interactions, particularly GAP association with p62.

Here we report that tyrosine phosphorylation of GAP stabilizes its association with p62 at membranes of cells transformed by v-Src. These findings suggest that one function of GAP phosphorylation is to regulate its interaction with p62, which in turn may contribute to regulation of Ras mitogenic signalling pathways.

MATERIALS AND METHODS

Cell Culture—Rat 3Y1 fibroblasts transformed by Rous sarcoma virus (SR-3Y1) (35) were maintained in Dulbecco’s modified Eagle’s medium containing iron-supplemented 5% bovine calf serum (Hyclone) as described earlier (31, 34). Dexamethasone (Sigma) treatment of cells was performed for 15–20 h and was used at a concentration of 300 nM.

Construction and Expression of KT3-tagged GAP—For introducing the 8-amino acid KT3 epitope (TTPFEPETP) (36) to the COOH terminus of bovine GAP, a double-stranded 55-base pair oligonucleotide was synthesized that encodes the extreme COOH-terminal 8 amino acids of GAP, with the KT3 epitope flanked by a NotI site at its 5’-end and a PUC stop codon plus EcoRI site at its 3’-end. The oligonucleotide was phosphorylated and cloned into the EcoRI and XbaI sites of pUC18 together with an XbaI-Acel GAP fragment (nucleotides 2947–3105 of the bovine GAP coding region) (37).

For efficient expression of bovine GAP cDNA in rat 3Y1 cells, a double-stranded 55-base pair oligonucleotide was synthesized consisting of the 5’-GAP coding sequence in addition to a modified 5’-translated sequence conforming to Kozak’s rules (38), flanked by a SalI site at its 5’-end and a NotI site at its 3’-end. The phosphorylated oligonucleotide was cloned into the BamHI and SalI sites of pUC18 together with a NotI-BamHI GAP fragment containing the sequences 64–2098 of bovine GAP. For regenerating the full-length GAP construct, the SalI-BamHI fragment and XbaI-EcoRI fragment cloned into pUC18 vectors described above were isolated and cloned into the SalI and EcoRI site of pUC18 together with a BamHI-XbaI GAP fragment (nucleotides 2068–2853 of bovine GAP) (modified sequences at both the 5’-end and 3’-end of the full-length GAP construct were confirmed by the dideoxy DNA sequencing method (39).

To generate full-length GAP(F457), a 601-base pair Psrl fragment of bovine GAP containing the codon for Tyr-457 was isolated and subcloned into pUC18. Site-specific mutagenesis was performed by the sequential polymerase chain reaction method (40) with Pfu polymerase (Stratagene), using two oligonucleotide primers incorporating TTT (Phe) instead of TAT (Tyr) plus universal reverse and forward primers. Amplified polymerase chain reaction products were further digested with PstI and BgIII and the isolated GAP fragment (nucleotides 1101–1548 of bovine GAP) subcloned into the full-length GAP in pUC18 DNA sequencing in the region of the polymerase chain reaction mutagenesis was performed to exclude the possibility of errors by the polymerase. Structures of the GAP recombinants described above are shown in Fig. 1.

Salt-EcoRI fragments containing the full-length GAP or GAP(F457) coding sequence from the above constructs were blunt-ended and cloned into Sall-restricted and blunt-ended pMAMneo (Clonetech) expression vector. Wild-type GAP and GAP(F457) pMAMneo constructs, and pMAMneo vector alone control, were transfected using the calcium phosphate coprecipitation method (41) into SR-3Y1 cells. G418-resistant colonies were picked and shown in Fig. 1. The clones 1–4 (as described above) were expanded.

Immunoprecipitation and Immunoblotting—Confluent 10-cm plates of SR-3Y1 cells (approximately 108 cells) were washed twice with cold phosphate-buffered saline containing 1 mM sodium orthovanadate, and then lysed in 1 ml of cold RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg leupeptin, 1 μg antipain, and 0.1 μg aprotinin). Lysates were heated at 100 °C for 5 min, cooled on ice, forced through a 26-gauge needle, and diluted 10-fold with RIPA buffer described above prior to immunoprecipitation.

Proteins resolved by SDS-PAGE were electrotransferred to Nitrocellulose and probed with the monoclonal antibody probes described (31, 34). Monoclonal antibody probes were detected using 10 μCi of 125I-labeled sheep anti-mouse IgG (ICN) followed by exposure to Kodak X-AR-5 film with an intensifying screen at −80 °C. For immunoblotting polyclonal antibodies, the bound antibodies were detected using 10 μCi of 125I-labeled affinity purified protein-A (Amersham). Quantitation of 125I-labeled IgG or protein-A binding to immunoblots was performed with a Molecular Dynamics PhosphorImager.

Subcellular Fractionation—Cells were scraped into cold phosphate-buffered saline containing 1 mM sodium orthovanadate and then collected by centrifugation. 8 g of cell pellets (wet weight) were resuspended with 3 ml of hypotonic buffer (10 mM Tris-Cl, pH 7.5, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 μg leupeptin, 1 μg antipain, and 0.1 μg aprotinin) (43); 10 min later, the cells were transferred to a Dounce homogenizer and disrupted by 40 strokes. The homogenate was centrifuged at 800 × g for 10 min to remove nuclei and then the postnuclear supernatant was centrifuged at 100,000 × g for 40 min. The supernatant fraction (S100) was adjusted to 1% Nonidet P-40. The pellet (P100) was gently rinsed with 3 ml of phosphate-buffered saline and then resuspended in 3 ml of hypotonic buffer containing 1% Nonidet P-40; 10 min later, insoluble materials were removed by centrifugation in a microcentrifuge for 5 min. The S100 and P100 fractions were immunoprecipitated with anti-KT3 antibodies and washed two times with cold RIPA buffer containing 1 mM sodium orthovanadate as described above.

In Vivo Labeling and Phosphopeptide Mapping Analysis—For metabolic labeling, SR-3Y1 cells in 10-cm dishes were grown to confluence and treated with dexamethasone prior to labeling. Cellular proteins in these cells were labeled with 5 μCi of [32P]orthophosphate (ICN) by incubation for 3 h at 37 °C in 2 ml of phosphate-free Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) supplemented with 0.5% fetal calf serum plus 50 μM sodium orthovanadate.

Labeled proteins separated by SDS-PAGE were transferred to nitrocellulose membranes and then localized by autoradiography. Immobilized proteins were directly digested with 20 μg of trypsin (Worthington) by incubating membrane pieces in 50 mM NaH2HCO3 (pH 7.3–7.6) at 37 °C for 4 h and tryptic peptides were then oxidized with performic acid. Tryptic peptides were analyzed by electrophoresis in SDS to remove (first dimension) and chromatography (second dimension) on TLC plates. All of the procedures involving peptide mapping analysis were performed according to previously described protocols (44, 45).

Antibodies—Anti-GAP(638) rabbit antiserum raised against amino acid residues 139–152 of bovine GAP (13) was generously provided by J. B. Gibbs (Merck Sharp & Dohme Research Laboratories). The murine monoclonal anti-phosphothreonine antibody (46) was obtained from UBI (Lake Placid, NY).

RESULTS

More Than 10% of Total Cellular GAP Is Tyrosine Phosphorylated in v-Src Transformed Rat Fibroblasts—Previous stud-
ies established that GAP is tyrosine phosphorylated in v-Src transformed cells (27, 30), although the stoichiometry of this modification has not been determined. To estimate the fraction of tyrosine-phosphorylated GAP in v-Src transformed SR-3Y1 fibroblasts, cell lysates were prepared using RIPA buffer for anti-PTyr immunoprecipitation followed by anti-GAP immunoblot analysis. This analysis revealed that only 1% of total cellular GAP was recovered by anti-PTyr antibodies (data not shown). Earlier studies demonstrated that tyrosine-phosphorylated GAP physically associates with two major phosphoproteins, p62 and p190, in v-Src transformed cells (27, 32, 43). If phosphorylation is important for these protein-protein interactions, phosphotyrosine in GAP could be inaccessible to anti-PTyr antibodies. Therefore, cells were lysed under denaturing conditions in boiling SDS buffer that disrupted GAP association with p190 and p62. Denatured lysates were subsequently diluted 10-fold in RIPA buffer prior to immunoprecipitation with anti-PTyr antibodies. The relative amount of GAP recovered by anti-PTyr antibodies was compared with the amounts of total cellular GAP from the same lysate by immunoblot analysis using anti-GAP antibodies as probe (Fig. 2). For maximum recovery of tyrosine-phosphorylated GAP, three consecutive immunoprecipitations of the same lysate were combined and analyzed (Fig. 2, lane 4). Results indicated that about 12% of total cellular GAP was recovered by anti-PTyr antibodies (Fig. 2, compare lanes 2, 3, and 4). The lysate supernatant after the third immunoprecipitation was further immunoprecipitated with anti-PTyr antibodies (Fig. 2, lane 5) to confirm that most tyrosine-phosphorylated GAP was recovered in the previous immunoprecipitations. As a control, three consecutive immunoprecipitations using anti-PTyr antibodies preincubated with phenylphosphate as competitor were combined and analyzed to demonstrate that tyrosine-phosphorylated GAP was specifically immunoprecipitated by anti-PTyr antibodies (Fig. 2, compare lanes 4 and 6). In several independent experiments, we observed that between 10 and 20% of endogenous cellular GAP in SR-3Y1 cells was recovered by anti-PTyr antibodies, indicating that at least 10% of cellular GAP is tyrosine phosphorylated in v-Src transformed rat fibroblasts.

**GAP<sup>Δ457</sup> Expressed in v-Src Transformed Rat Fibroblasts**

*Lacks a Phosphopeptide That Contains Tyr-457—Previous studies suggested that Tyr-457 in bovine GAP is the major site phosphorylated in *vitro* and *in vivo* by the v-Src and epidermal growth factor receptor kinases (33, 34). To confirm that Tyr-457 in GAP is the major phosphorylation site in v-Src transformed cells, full-length GAP containing Phe-457 instead of Tyr-457 was constructed as described under “Materials and Methods.” At the COOH terminus of each GAP construct, an 8-amino acid KT3 epitope corresponding to the COOH terminus of SV40 large T-antigen (36) was appended to facilitate immunoprecipitation of exogenously expressed GAP by anti-KT3 monoclonal antibody (Fig. 1). These GAP constructs were introduced into v-Src transformed cells and stable cell lines were established that expressed from 1.5- to 2-fold more exogenous GAP than endogenous GAP in response to dexamethasone. No changes in cell morphology were apparent in these v-Src transformed cells irrespective of GAP induction (data not shown).

Lysates from each stably transfected cell line incubated with or without dexamethasone were prepared for immunoprecipitation with anti-KT3 antibodies followed by immunoblot analysis using anti-GAP antibodies as probe. KT3-tagged GAP or GAP<sup>Δ457</sup> was inductively expressed by dexamethasone, although a low level of KT3-tagged GAP was expressed in the absence of dexamethasone (Fig. 3A). No endogenous GAP was immunoprecipitated from the control cell lysate by anti-KT3 antibodies,

**Fig. 2. Stoichiometry of tyrosine phosphorylation of endogenous GAP in v-Src transformed rat 3Y1 cells.** Whole-cell lysates (WCL) of v-Src transformed 3Y1 cells (SR-3Y1) were prepared in boiling 0.5% SDS buffer. For immunoprecipitation, cell lysates were diluted 10-fold in RIPA buffer lacking SDS, and then proteins were immunoprecipitated three times with anti-PTyr antibodies preincubated without or with 10 μM phenylphosphate (lanes 4 and 6, respectively). The supernatant after the third immunoprecipitation was immunoprecipitated once more with anti-PTyr antibodies (lane 5). Proteins recovered by anti-PTyr antibodies were combined and analyzed by immunoblot using anti-GAP(658) antibodies as probe followed by affinity-purified <sup>125</sup>I-labeled protein A (lanes 4–6). For comparison, 5, 10, and 15% aliquots of the undiluted, whole lysate prior to immunoprecipitation were directly analyzed on the same blot (lanes 1–3).

**Fig. 3. In *vivo* phosphopeptide mapping of the KT3-tagged GAP or GAP<sup>Δ457</sup> expressed in v-Src transformed rat 3Y1 cells.** SR-3Y1 cells stably transfected with pMAMneo vector, or wild-type GAP or GAP<sup>Δ457</sup> constructs derived from this vector, were untreated or treated with 300 nm dexamethasone for 15 h prior to preparing cell lysates and labeling with <sup>32</sup>Porthophosphate. A, unlabeled cell lysates were immunoprecipitated with anti-KT3 antibodies, and then analyzed by immunoblot using anti-GAP(658) antibodies as probe followed by affinity-purified <sup>125</sup>I-labeled protein A. B, cells were metabolically labeled with <sup>32</sup>Porthophosphate for 3 h. Labeled KT3-tagged GAP proteins were isolated by immunoprecipitation with anti-KT3 antibodies, separated by SDS-polycrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. GAP proteins were localized by autoradiography, and then tryptic phosphopeptides were prepared as described under “Materials and Methods.” Tryptic phosphopeptides were analyzed on TLC plates by electrophoresis at pH 1.9 in the horizontal dimension with the anode on the left, and ascending chromatography in the vertical dimension. The origin is marked with an arrow, and the phosphopeptides derived from GAP are labeled A and B.
demonstrating that anti-KT3 antibody did not cross-react with endogenous GAP (Fig. 3A, lanes 1 and 2).

To determine whether GAPFK457 lacks the major phosphorylation site in v-Src transformed cells, each stably transfected cell line was metabolically labeled with [32P]orthophosphate in the presence of dexamethasone. Immunoprecipitates prepared from these cell lysates with anti-KT3 antibodies contained phosphorylated GAP or weakly phosphorylated GAPFK457 (data not shown; see below). [32P]-Labeled GAP or GAPFK457 was isolated, digested with trypsin, and analyzed by two-dimensional phosphopeptide mapping. As expected from previous studies (34), wild-type GAP contained two tryptic phosphopeptides, A and B, but GAPFK457 contained only tryptic phosphopeptide B (Fig. 3B). Because our earlier studies established that phosphopeptide A contains Tyr-457 as the only phosphorylation site (34), these results confirm that Tyr-457 is the major in vivo phosphorylation site in v-Src transformed cells. Other phosphopeptides that are variably detected appear to arise from phosphoproteins nonspecifically immunoprecipitated by anti-KT3 antibodies because these phosphopeptides are not observed using in vivo labeled endogenous GAP immunoprecipitated by anti-GAP antibodies (data not shown).

Binding of p62 to GAPFK457 Is Reduced Compared with Wild-type GAP—Tyr-457 in bovine GAP is located immediately COOH-terminal to the second SH2 region (Fig. 1), suggesting that phosphorylation of this site might regulate protein-protein interactions, particularly GAP association with p62. To test this hypothesis, v-Src transformed rat 3Y1 cells stably transfected with vector, GAP, or GAPFK457 constructs were treated with dexamethasone to induce KT3-tagged GAP expression prior to preparing cell lysates. Immunoprecipitates prepared with anti-KT3 antibodies were analyzed directly by immunoblot using either anti-PTyr or anti-GAP antibodies as probes (Fig. 4). Similar amounts of KT3-tagged GAP were present in lysates from cells expressing GAP or GAPFK457, but none was detectable in control cell lysate (Fig. 4, bottom panel). As expected from the in vivo phosphopeptide mapping experiments described above, tyrosine phosphorylation of GAPFK457 was significantly reduced because it lacked the major phosphorylation site (Fig. 4, top panel). Nevertheless, the overall levels of p90 coimmunoprecipitated with GAP or GAPFK457 were not substantially changed, indicating that association of GAP with p90 did not involve Tyr-457 phosphorylation of GAP (Fig. 4, top panel). In some experiments, however, we variably observed that GAPFK457 associated with more p190 than did wild-type GAP (data not shown). In contrast, we reproducibly found that the amounts of p62 coimmunoprecipitated with GAP correlated with the degree of GAP tyrosine phosphorylation (Fig. 4), suggesting that Tyr-457 phosphorylation of GAP is important for its association with p62 protein. Quantitation shown in Table I using a PhosphorImager indicated that the relative binding of p62 to GAPFK457 decreased approximately 3-fold compared with tyrosine-phosphorylated wild-type GAP.

Binding of p62 to GAPFK457 Is Greatly Reduced at Cell Membranes—Previous studies established that a substantial fraction of tyrosine-phosphorylated GAP is localized at cell membranes in response to growth factor stimulation or in v-Src transformed cells (26, 43). To determine whether GAP-p62 complexes are localized to membranes as a function of Tyr-457 phosphorylation in GAP, cell lysates were separated into membrane and cytoplasmic fractions. Immunoprecipitates prepared with anti-KT3 antibodies from each fraction were analyzed directly by immunoblot using either anti-PTyr or anti-GAP antibodies as probes (Fig. 5A). The amounts of wild-type GAP (~25%) associated with cell membranes were reproducibly more than GAPFK457 (~15%) (Fig. 5, A, bottom panel, and B), indicating that localization of GAPFK457 to membranes is partially defective. Tyrosine-phosphorylated wild-type GAP is distributed more in the particulate fraction than the soluble fraction, while GAPFK457 phosphorylated at the minor site of tyrosine phosphorylation is present predominantly in the soluble fraction, indicating that Tyr-457-phosphorylated GAP is enriched at cell membranes (Fig. 5A, top panel). In repeated experiments, we observed that some tyrosine-phosphorylated proteins comigrating with GAP were nonspecifically immunoprecipitated by anti-KT3 antibodies (data not shown). Therefore, although the anti-PTyr immunoblot reflects the distribution of tyrosine-phosphorylated GAP, this analysis does not show the absolute amounts of tyrosine-phosphorylated GAP.

Wild-type GAP-associated p62 was distributed slightly more in the particulate fraction than soluble fraction (Fig. 5, A, top panel, and B), consistent with a previous report (43). Subcellular distribution of the wild-type GAP-associated p62 corresponded to the distribution of tyrosine-phosphorylated GAP rather than total cellular GAP (Fig. 5A, compare lanes 3 and 4), suggesting that Tyr-457 phosphorylation of GAP enhances GAP

TABLE I

| Experiment 1 | Experiment 2 | Experiment 3 |
|--------------|--------------|--------------|
| GAPFK457    | 100          | 100          | 100          |
| GAPFK457    | 34           | 38           | 33           |

* Each of the three independent experiments was performed as described in the legend to Fig. 4. Quantitation of 125I-labeled IgG or protein A bound to immunoblots was done with a PhosphorImager. The amount of GAP-associated p62, quantitated with antiphosphotyrosine antibodies, was normalized to the amount of GAP, quantitated with anti-GAP antibodies, prior to calculating the relative percentage of p62 bound to GAP. The amounts of wild-type GAP-associated p62 were assumed to be 100%.
association with p62. In striking contrast, we reproducibly found that the amounts of GAP\[^{F457}\] associated p62 were severely reduced at cell membranes and enriched in the soluble fraction (Fig. 5, A, top panel, and B). These results indicate that Tyr-457 phosphorylation of GAP stabilizes its interaction with p62 at cell membranes. Unlike p62, p190 associated with wild-type GAP or GAP\[^{F457}\] was predominantly in the cytoplasmic fraction (Fig. 5A, top panel, lanes 4 and 6). Subcellular distribution of p190 corresponds to the distribution of total cellular GAP (Fig. 5A), suggesting that p190 associates mainly with unphosphorylated GAP. The amounts of GAP\[^{F457}\]-associated p190 in the cytoplasmic fraction were detectably more than wild-type GAP-associated p190 (Fig. 5A, compare lanes 4 and 6). These results suggest that the stoichiometry of p190-GAP complexes may be influenced by reduced p62-GAP association.

**DISCUSSION**

Previous studies showed that GAP is one of the proteins that is tyrosine phosphorylated in v-Src transformed cells (27, 30). In addition, our *in vitro* reconstitution experiments using a baculovirus expression system demonstrated that GAP is a direct substrate for the v-Src tyrosine kinase (31). Because GAP is a key regulator of Ras, which is implicated in mitogenic signalling pathways downstream of v-Src (8), these findings suggest that GAP might be a physiologically relevant substrate for the v-Src tyrosine kinase. In this study, we measured the approximate stoichiometry of tyrosine-phosphorylated GAP in v-Src transformed rat fibroblasts. Using a denatured lysate procedure with anti-PTyr antibodies and appropriate controls, we determined that more than 10% of total cellular GAP is tyrosine phosphorylated in these v-Src transformed cells. However, this is only a minimum estimate because phosphotyrosine could be unstable during cell lysis and the anti-PTyr antibodies might not react with all of the phosphorylated GAP. Other studies using platelet-derived growth factor-stimulated NIH 3T3 cells indicated that the stoichiometry of tyrosine-phosphorylated GAP was approximately 30% in those cells (47). These results suggest that a substantial fraction of cellular GAP is a direct target for activated tyrosine kinases, consistent with a significant physiologic role of tyrosine phosphorylation in GAP.

Our earlier studies suggested that 2 tyrosine residues in GAP are phosphorylated in v-Src transformed cells, and that one of these is Tyr-457 (of bovine GAP) which is directly phosphorylated by v-Src (34). The other unidentified tyrosine phosphorylation site is minor and appears to be phosphorylated by another kinase present in v-Src transformed cells (34). Our phosphopeptide mapping experiments of GAP\[^{F457}\] reported here confirm that Tyr-457 of bovine GAP is the major *in vivo* phosphorylation site in v-Src transformed cells. Significantly, the equivalent of Tyr-457 in human GAP is specifically phosphorylated in cells stimulated by epidermal growth factor (33), providing further support for the suggestion that this modification is physiologically relevant.

GAP has two SH2 domains and one SH3 domain, in the order SH2-SH3-SH2. Previous studies using the NH2-terminal GAP SH2 domain (SH2-N) expressed in bacteria indicated that GAP undergoes SH2-mediated interactions with the epidermal growth factor receptor, platelet-derived growth factor receptor, and p62 (24, 25). Extensive mutagenesis in the GAP associated p62 (bottom panel), were quantitated by using a Phosphor-Imager. For comparison, the relative amounts of proteins in either particulate or soluble fractions are indicated by percentage. Solid bar represents GAP or associated p62 in the particulate fraction and hatched bar indicates GAP or associated p62 in the soluble fraction. Each data set represents the mean of three separate experiments ± S.D.
SH2-N domain demonstrated that highly conserved basic amino acids are important for p62 binding to GAP in vitro (48). These results suggest that the SH2-N domain in GAP is a binding site for p62. In comparison to the GAP SH2-N domain, the GAP SH2-C domain binds weakly to tyrosine-phosphorylated proteins in vitro (24, 25). While the role of the GAP SH2-C domain is not clear, it has been implicated in cooperative binding of both GAP SH2 domains to tyrosine-phosphorylated proteins in vivo (24, 25, 48). The finding that Tyr-457 in bovine GAP is located immediately COOH-terminal to the SH2 domains led us to postulate that this specific phosphorylation might regulate GAP association with p62. Our results presented here suggest that the binding of p62 to the GAPF457 mutant, which lacks the major phosphorylation site, was decreased approximately 3-fold compared with wild-type GAP in v-Src transformed cells. Because p62 was detected by anti-PTyr antibodies, however, these data do not distinguish between decreased p62 bound to GAP or decreased phosphorylation of GAP-associated p62. One role of tyrosine phosphorylation in GAP, therefore, might be to increase its binding affinity for tyrosine-phosphorylated p62 by modulating the SH2-SH3-SH2 region conformation in GAP. Other studies have suggested that, in murine fibroblasts overexpressing c-Src, GAP is not tyrosine phosphorylated and yet it associates with p62 (49). These results are consistent with our finding that blocking GAP phosphorylation does not completely abolish its association with p62, indicating that some other factor(s) also contributes to their interaction.

Previous studies using subcellular fractionation procedures established that GAP is almost entirely cytosolic in normal cells, and activated tyrosine kinases induce a redistribution in GAP subcellular localization to membranes (26, 43). However, it is difficult to determine whether p190 and p62 associate with the cytoplasmic tail of the GAP protein. Our studies have determined that p190 binds to the cytoplasmic tail of GAP, consistent with previous reports that p190 binds to the cytoplasmic tail of GAP. This binding is thought to be mediated by the p190's SH2 domain, which binds to the tyrosine-phosphorylated tail of GAP.

Activation of GAP by activated tyrosine kinases in vivo induces gene expression in a Ras-dependent manner (50) may support the suggestion of an effector role for the p62-GAP complex in mitogenic signal transduction. A cDNA encoding p62 has been cloned and the predicted protein shares extensive sequence similarity with an hnRNP protein (51). Because our results suggest that the association of p62 with GAP is enhanced by GAP tyrosine phosphorylation at cell membranes, further investigation of p62 function may provide new insights into Ras-mediated signal transduction.

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