Activated Src Tyrosine Kinase Phosphorylates Tyr-457 of Bovine GTPase-activating Protein (GAP) in Vitro and the Corresponding Residue of Rat GAP in Vivo*

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GTPase-activating protein (GAP) is a key regulator of the cellular Ras protein, which is implicated in oncogenic signal transduction pathways downstream of the viral Src (v-Src) kinase. Previous studies demonstrated that v-Src induces tyrosine phosphorylation of GAP, suggesting that GAP may provide a biochemical link between v-Src and Ras signaling pathways. To determine the precise residues in GAP phosphorylated by Src kinases, we used a baculovirus/insect cell expression system for investigating in vitro phosphorylation of GAP. Phosphopeptide mapping analysis revealed that v-Src and normal cellular Src (c-Src) phosphorylate tyrosine residues in bovine GAP at one major site and one minor site in vitro. Significantly, the major site of GAP phosphorylation in vitro is also the major site of in vivo tyrosine phosphorylation of GAP in rat fibroblasts transformed by v-Src. Analyses of GAP deletion mutants and TrpE-GAP fusion proteins established that Tyr-457 of bovine GAP (and the corresponding residue of rat and human GAP) is the major site of tyrosine phosphorylation. Our results demonstrate that the v-Src kinase induces phosphorylation of the same tyrosine residue of GAP in vitro and in vivo, suggesting that GAP is a direct substrate of activated Src kinases in vivo. Because epidermal growth factor receptor phosphorylates the equivalent tyrosine residue in human GAP (Tyr-460), these findings are consistent with the hypothesis that specific phosphorylation of GAP at this site may have a physiologically important role in regulating mitogenic Ras signaling pathways.

The src oncogene encodes a membrane-localized tyrosine-specific protein kinase whose enzymatic activity is necessary for its ability to induce oncogenic transformation (1–3). Although normal cellular Src (c-Src) is tightly regulated in its kinase activity relative to oncogenic viral Src (v-Src), mutations in c-Src protein that elevate its enzymatic activity also enhance its oncogenic potential (4, 5). Because oncogenicity correlates with kinase activity, the Src oncoprotein may induce transformation by phosphorylating critical cellular proteins that control cell proliferation (6). Cells expressing v-Src and activated c-Src contain a large number of tyrosine phosphorylated proteins, many of which have been characterized (3, 6). However, physiologically relevant substrates with causal roles in signal transduction pathways leading to transformation have not been identified.

One of the proteins that is tyrosine-phosphorylated in cells transformed by v-Src is the Ras GTPase-activating protein (GAP)1 (7). GAP is a regulator of Ras protein (8–10), which has an essential role in mitogenic signaling pathways downstream of receptor and oncogenic tyrosine kinases, including v-Src (11, 12). GAP interacts with cellular Ras in the active GTP-bound form and stimulates its intrinsic ability to hydrolyze GTP to GDP, thereby inactivating Ras (8–10). This negative regulatory function of GAP is consistent with the recent finding that overexpression of human GAP blocks transformation by cellular Ras (13). In addition to a regulatory function, GAP may also be required for Ras effector function, possibly as the downstream target molecule of Ras (14, 15). Several observations suggest that GAP may have a cardinal role in oncogenesis by activated tyrosine kinases, including v-Src. First, GAP is phosphorylated on tyrosine in cells transformed by v-Src and other oncogenic tyrosine kinases (7). Second, activation of the oncogenic potential of normal c-Src by mutation correlates with increased tyrosine phosphorylation of GAP (16). Third, GAP is physically associated in complexes with, and phosphorylated by, both c-Src and v-Src (17, 18). Finally, cells transformed by v-Src and other oncogenic tyrosine kinases contain elevated levels of activated Ras bound to GDP (19, 20), and overexpression of GAP inhibits transformation by v-Src (21, 22).

How tyrosine kinases might regulate GAP activity is not clear. Phosphorylation of GAP by activated tyrosine kinases may directly inhibit its ability to stimulate the Ras GTPase activity, thus keeping cellular Ras preferentially in the active GTP-bound state. Alternatively, GAP activity may be modulated by allosteric interactions with regulatory proteins in a complex; activated tyrosine kinases induce GAP to form multiple heteromeric complexes with other tyrosine-phosphorylated proteins, notably 62- and 190-kDa proteins (7, 23, 24). Another possibility is that tyrosine phosphorylation of GAP might stimulate its putative effector function (14, 15). This question of how tyrosine phosphorylation of GAP regulates its activity could be addressed more directly by identifying the sites in GAP that are phosphorylated by activated tyrosine kinases. We described previously (18) in vitro reconstitution of complexes containing GAP and the Src kinases using a

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1 The abbreviations used are: GAP, GTPase-activating protein; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; mAb, monoclonal antibody; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
baculovirus/insect cell expression system. Here we report that Tyr-457 of bovine GAP is the major tyrosine residue phosphorylated in this in vitro system by both v-Src and c-Src. In addition, the same tyrosine residue of rat GAP is phosphorylated in fibroblasts transformed by v-Src. This residue corresponds to Tyr-460 of human GAP that is phosphorylated by the epidermal growth factor receptor kinase (25). These findings raise the possibility that phosphorylation of this specific tyrosine residue in GAP might contribute to signal transduction by both normal growth factor receptor and oncogenic tyrosine kinases.

MATERIALS AND METHODS

Cell Culture—Spodoptera frugiperda (SF9) insect cells (American Type Culture Collection) were grown at 27°C without CO2 in Grace's insect cell medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum, 3.3 g/liter yeastolate, 3.3 g/liter lactalbumin, 50 μg/ml gentamicin, and 2.5 μg/ml G418 (as described (26, 27). The rat 3Y1 cells or rat 3Y1 cells transformed by v-Src (SR-3Y1) were maintained in Dulbecco's modified Eagle's medium containing 5% bovine calf serum as described earlier (28).

Construction and Expression of Recombinant Baculoviruses and TrpE-GAP Fusion Vectors—Construction of the following recombinant baculoviruses has been described (18): bc-Src encoding the full-length of normal chicken c-Src, bv-Src encoding Rous sarcoma virus v-Src, bGAP encoding the full-length of bovine GAP, bGAPSH encoding bovine GAP lacking amino acids 1-171, and bGAPDC encoding bovine GAP lacking amino acids 172-520. For expression of these recombinant baculoviral proteins, 2 × 10^6 Sf9 cells in 25-cm² flasks were routinely infected with the designated virus at a multiplicity of infection of 10 and lysed 48-h post-infection. All of the procedures involving baculoviruses were performed according to previously described protocols (26, 27).

Construction of the TrpE-GAPSH2 vector encoding residues 171–448 of human GAP and the TrpE-GAP vector encoding residues 171–520 of human GAP has been described earlier (25). TrpE-GAPSH(460) is identical to TrpE-GAP except that it contains Phe-460 substituted for Tyr-460 by site-directed mutagenesis (25). RRL bacteria containing the appropriate TrpE-fusion vectors were grown for 4 h in modified M9 medium supplemented with tryptophan, transferred into 50 ml of the same medium lacking tryptophan, and further cultivated for 2 h to induce TrpE-fusion protein expression.

The tryptophan analog indoleacrylic acid was added to the culture for 4 h as an additional means of induction (29).

Metabolic Labeling—Rat 3Y1 fibroblasts seeded in 10-cm dishes were grown to confluence prior to labeling. Cellular proteins from these cells were labeled with 2 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 (GIBCO) supplemented with 0.5% fetal calf serum plus 50 mM sodium orthovanadate. Immunoprecipitations—Infected Sf9 cells were washed twice with cold phosphate-buffered saline containing 1 mM sodium orthovanadate and then lysed in 1 ml of cold lysis buffer (50 mM NaCl, 50 mM Tris-CI, pH 7.5, 1% Nonidet P-40, 0.25% sodium deoxycholate, 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml a2-macroglobulin, 1 μM leupeptin, 1 μM antipain, and 0.1 μM aprotinin). Lysates were clarified by centrifugation in a microcentrifuge for 15 min at 4°C. For in vitro reconstitution of Src-GAP complexes, lysates were mixed with each other for 3 h at 4°C and then proteins were immunoprecipitated by incubating with monoclonal anti-Src antibody (mAb 2-17) for 1 h at 4°C as described previously (18). In some experiments, Sf9 cells were coinfected with viruses encoding both GAP and Src proteins prior to cell lysis. Protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) was then added for 30 min, and immunoprecipitates were washed by pelleting in cold lysis buffer without protease inhibitors.

A pellet from 50 ml of RRL bacteria expressing TrpE-GAP fusion proteins was lysed in 1 ml of cold bacterial lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.3 M NaCl, 1% Nonidet P-40, 3 mg/ml lysozyme) on ice for 2 h and then sonicated. Cell lysates were clarified by centrifugation in a microcentrifuge for 15 min at 4°C. Clarified soluble fractions of these cell lysates containing bacterial GAP fusion proteins were mixed with clarified Sf9 cell lysates containing baculovirus-expressed Src on ice for 3 h in order to form Sf9/TrpE-GAP protein complexes. Mixed cell lysate containing Sf9/TrpE-GAP protein complexes was immunoprecipitated with anti-Src antibody (mAb 2-17) as described above.

Lysis of rat fibroblasts and immunoprecipitation of proteins from these cells was performed as described above for Sf9 cells except that cellular proteins from 50 ml of Sf9 cultures were lysed in 1 ml of cold lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2) with 20 μCi of [γ-32P]ATP (Amersham Corp.). Labeled reaction products were boiled for 5 min in 0.5% SDS buffer, diluted 1:10 in lysis buffer, and immunoprecipitated with the indicated antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE, and dried gels were autoradiographed for 30 min at room temperature using Kodak XAR-5 film.

For in vitro phosphorylation of the synthetic GAP peptide (EYINTR), 1 μg of peptide was added to 30 μl of kinase reaction buffer containing baculovirus-expressed v-Src that had been immunoprecipitated and washed extensively from a clarified lysate of infected Sf9 cells. After incubation with 20 μCi of [γ-32P]ATP, the supernatant containing phosphorylated peptide was spotted on a thin-layer cellulose (TLC) plate followed by two-dimensional separation for purification of the phosphorylated synthetic peptide as described before (30).

Peptide Mapping and Phosphoamino Acid Analysis—Labeled proteins separated on a 10% SDS-polyacrylamide gel were transferred to nitrocellulose and then localized by autoradiography. Immobilized proteins were directly digested with trypsin (20 μg) by incubating membrane pieces in 50 mM NH4HCO3, pH 7.3–7.6, at 37°C for 4 h. Tryptic peptides were then oxidized with performic acid and subjected to both peptide mapping and phosphoamino acid analysis. For peptide mapping, tryptic peptides were analyzed by electrophoresis in pH 1.9 buffer (first dimension) and chromatography (second dimension) on TLC plates as described (30). For phosphoamino acid analysis, tryptic peptides were hydrolyzed in 6 M HCl at 100°C for 24 h. The hydrolysates were analyzed by two-dimensional electrophoresis in pH 1.9 buffer (first dimension) and pH 5.5 buffer (second dimension) on TLC plates. To elute the phosphate-containing peptides from TLC plates, the areas on TLC plates were aspirated using 1-ml blue pipette tips plugged by 6.5-mm polystyrene disks (Omnifit) and eluted in pH 1.9 buffer. Digestes of the eluted tryptic peptides with chymotrypsin, thermolysin, and V8 protease were carried out in 50 mM NH4HCO3, pH 7.3–7.6, at 37°C for 4 h. All of the procedures involving peptide mapping and phosphoamino acid analysis were performed according to previously described protocols (30, 31).

Antibodies—Anti-GAP(368) antisera raised against amino acid residues 139–152 of bovine GAP (32) was generously provided by J. H. R. and M. R. Merrick Sharp (Lederle Research Laboratories). Monoclonal anti-Src antibodies (mAb 2-17) raised against amino acid residues 2–17 of Src were obtained from Microbiological Associates (Bethesda, MD). Monoclonal anti-TrpE antibodies were obtained from Oncogene Science (Manhasset, NY).

RESULTS

v-Src Phosphorylates 2 Tyrosine Residues of GAP in Vitro—Our previous studies (18) with recombinant baculovirus-expressed proteins showed that GAP serves as substrate for both the v-Src and c-Src kinases in vitro or in insect cells. In addition, deletion of the GAP amino-terminal portion containing SH2 regions diminished GAP phosphorylation and association with Src (18). We used this baculovirus expression system to investigate the tyrosine residues in GAP phosphorylated in vitro by Src. For reconstituting Src-GAP complex in vitro, lysates of v-Src-infected Sf9 cells were mixed with lysates of Sf9 cells infected with either bGAP, bGAPSH, or bGAPDC (Fig. 1). Src and its associated GAP or GAP deletion mutants were then immunoprecipitated with anti-Src mAb 2-17 and incubated with [γ-32P]ATP in an in vitro kinase reaction. Small aliquots of the total phosphorylated products were analyzed directly by SDS-PAGE and autoradiography to compare overall levels of v-Src kinase activities (Fig. 2A). Because some insect cell or baculoviral proteins present in the immunoprecipitates served as sub-
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FIG. 1. Structures of bovine GAP, deletion mutants of bovine GAP, and TrpE-GAP fusion proteins. For each protein, the amino acid positions are numbered relative to the first amino acid of GAP (designated +1). Hatched and dotted boxes represent the SH2 and SH3 regions, respectively, of both bovine GAP and human GAP (34). The solid box indicates the catalytic domain of bovine GAP (32, 34). For bGAPdC and bGAPdSH, the regions deleted from bovine GAP are denoted by open spaces at the indicated amino acid positions. For TrpE-GAP fusion proteins, the part of human GAP expressed as a fusion with TrpE protein is denoted by the indicated amino acid positions. Tyr-457 of bovine GAP is indicated together with its equivalent residue, Tyr-460, of human GAP. The difference in numbering of amino acids between human GAP and bovine GAP is primarily due to changes in the amino-terminal portions of these proteins (34).

FIG. 2. In vitro phosphorylation of GAP or GAP deletion mutants by v-Src. Lysates were prepared from S99 cells infected with bv-Src, bGAP, bGAPdSH, or bGAPdC. Lysates containing similar amounts of GAP or GAP deletion mutants (see C) were mixed with lysates containing equal amounts of baculovirus expressed v-Src. A, proteins from mixed cell lysates were immunoprecipitated with anti-Src mAb 2-17 antibodies and then incubated in an in vitro kinase reaction with [32P]ATP. Small aliquots (5%) of the total reaction products were analyzed directly by SDS-PAGE and autoradiography. B, remaining portions of [32P]-labeled reaction products were boiled in 0.5% SDS, diluted 10-fold in lysis buffer and then immunoprecipitated with anti-GAP(638) antibodies. These anti-GAP immunoprecipitates were then analyzed by SDS-PAGE and autoradiography. C, a fraction of each lysate containing GAP or GAP deletion mutants, prior to mixing with bv-Src-infected cell lysate, was separately analyzed by immunoblot using anti-GAP(638) antibodies as probe.

FIG. 3. Tryptic phosphopeptide maps of GAP or GAP deletion mutants labeled in vitro. [32P]-Labeled GAP or GAP deletion mutants resolved by SDS-PAGE as described in the legend to Fig. 2 were electrophoretically transferred to nitrocellulose membranes. Phosphorylated GAP or GAP deletion mutants immobilized on nitrocellulose were digested with trypsin and subjected to phosphopeptide mapping analysis. 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 are labeled a and b. A, tryptic phosphopeptide map of baculovirus-expressed GAP phosphorylated in vitro by baculovirus-expressed v-Src. B, tryptic phosphopeptide map of baculovirus-expressed GAPdSH phosphorylated in vitro by baculovirus-expressed v-Src. C, tryptic phosphopeptide map of baculovirus-expressed GAPdC phosphorylated in vitro by baculovirus-expressed v-Src. D, tryptic phosphopeptide map of rat GAP phosphorylated in vitro in a complex containing v-Src immunoprecipitated from transformed SR-3Y1 cells. E, mix of A and D. F, tryptic phosphopeptide map of baculovirus-expressed GAP phosphorylated in vitro by baculovirus-expressed c-Src.
insect cells due to low levels of phosphorylation at the regulatory Tyr-527 residue (Fig. 3F) (33).

Initial experiments using the amount of GAPdSH protein equivalent to that of GAP or GAPdC protein did not yield sufficient labeled phosphotryptic peptides for analysis. Therefore, we overexpressed a greater than 10-fold excess of GAPdSH protein with v-Src to detect efficient phosphorylation of GAPdSH (data not shown). Peptide mapping indicated that the GAPdSH protein contains only one tryptic phosphopeptide (Fig. 3B). When the tryptic phosphopeptides from both GAPdSH and wild type GAP were mixed and analyzed simultaneously, it was evident that the tryptic phosphopeptide from GAPdSH is phosphopeptide b (Fig. 4, A–C). In addition, both phosphopeptides a and b contain only phosphotyrosine (Fig. 4D). We conclude that GAPdSH lacks a major in vitro tyrosine phosphorylation site which is contained in phosphopeptide a. Therefore, this phosphopeptide must be present between amino acids 166 and 518 of bovine GAP that are deleted in GAPdSH protein (Fig. 1).

**Tryptic Phosphopeptide a is Present in Metabolically Labelled GAP**—To study GAP phosphorylation in vivo, SR-3Y1 rat fibroblast cells transformed by v-Src were metabolically labeled for 3 h with [32P]orthophosphate in the presence of the phosphatase inhibitor, sodium orthovanadate. Normal rat 3Y1 fibroblast cells were also labeled with [32P], in the presence of sodium orthovanadate for comparison with SR-3Y1 cells. Immunoprecipitates prepared from clarified cell lysates with anti-GAP(638) antibody contained phosphorylated GAP and other phosphoproteins, including p62 and p190 (Fig. 5A).

Phosphoamino acid analysis of GAP from normal rat 3Y1 fibroblast cells indicated that most phosphorylation of GAP occurred on serine residues (Fig. 5B). In contrast, GAP from SR-3Y1 rat cells transformed by v-Src contained phosphorylated tyrosine and phosphoserine (Fig. 5C). To compare GAP phosphorylation in vivo with GAP phosphorylation in vitro, [32P]-labeled GAP from SR-3Y1 cells was isolated, digested with trypsin, and analyzed by two-dimensional peptide mapping. In vivo labeled GAP contained two tryptic phosphopeptides (A and C) (Fig. 5D). When tryptic phosphopeptides from both in vivo labeled GAP and in vitro labeled GAP were mixed and analyzed simultaneously, phosphopeptide A from in vivo labeled GAP comigrated with phosphopeptide a from in vitro labeled GAP (Fig. 5E). Peptide b from the in vitro map had no counterpart from the in vivo map. Furthermore, peptide C from the in vivo map had no counterpart from the in vitro map.

To investigate whether the peptides from in vivo labeled GAP contain phosphothreonine or phosphoserine, we purified each peptide from TLC plates and subjected them to phosphoamino acid analysis. Results indicated that both peptide A and peptide C from in vivo labeled GAP contain phosphothreonine (data not shown). Because phosphoamino acid analysis of in vivo labeled GAP from v-Src-transformed cells indicated that there is proportionally more phosphoserine than phosphotyrosine, numerous weak tryptic phosphopeptides that appeared only in longer exposures most probably contain phosphoserine (data not shown). These results suggest that GAP contains multiple sites of serine phosphorylation in 3Y1 rat fibroblast cells. This is consistent with our observation that in vivo labeled GAP from normal rat 3Y1 cells showed barely detectable tryptic phosphopeptides, although it contained phosphoserine in phosphoamino acid analysis (data not shown). We conclude that peptide a/A contains the major tyrosine phosphorylation site of GAP both in vitro and in vivo.

**Tryptic Phosphopeptide a Is Located between Residues 448 and 520 in Human GAP**—To further define the major tyrosine phosphorylation site of GAP, we expressed two bacterial TrpE fusion proteins, designated TrpE-GAP-SH2, which contains human GAP residues 171–448 (corresponding to bovine GAP.
residues 168–445) and TrpE-GAPp, which contains human GAP residues 171–520 (corresponding to bovine GAP residues 168–517) (Fig. 1). To form complexes containing the baculovirus-expressed v-Src kinase and bacterial-expressed TrpE fusion proteins, equivalent amounts of bv-Src-infected Sf9 cell lysates were mixed with clarified bacterial cell lysate containing approximately equal amounts of the TrpE fusion proteins, TrpE-GAP-SH2 or TrpE-GAPp. Immunoblot analysis with anti-TrpE monoclonal antibody of immunoprecipitates prepared with anti-Src mAb 2-17 from these mixed cell lysates indicated that TrpE-GAP-SH2 and TrpE-GAPp stably associated with baculovirus-expressed v-Src, whereas the TrpE control protein did not (data not shown).

Immunoprecipitates prepared with anti-Src mAb 2–17 from mixed cell lysates were incubated with \[^{32}P\]ATP and then small aliquots of the reaction products were analyzed by SDSPAGE and autoradiography to compare total kinase activities of v-Src (Fig. 6A). To examine phosphorylation of GAP sequences specifically, TrpE-fusion proteins were further immunopurified using anti-TrpE antibodies. Results indicated that the baculovirus-expressed v-Src phosphorylated TrpE-GAPp but not TrpE-GAP-SH2 or TrpE protein (Fig. 6B). Tryptic phosphopeptide mapping analysis of TrpE-GAPp revealed only one major phosphopeptide, which corresponds to phosphopeptide a obtained from full-length bovine GAP phosphorylated \textit{in vitro} by v-Src (Fig. 6, C–E). This result suggests that this tyrosine phosphorylation site should be located between residues 448 and 520 of human GAP because v-Src phosphorylated TrpE-GAPp but not TrpE-GAP-SH2 (Fig. 1).

There are only four theoretical tryptic peptides which contain at least 1 tyrosine in the region of amino acids 448–520 of human GAP (corresponding to residues 445–517 of bovine GAP): EIYNTIR, DAFYK, GYLLK, and NLYFILEGSDAQLIY. Hence, these four tryptic peptides have different susceptibilities to chymotrypsin, thermolysin, and v8 protease digestion. To determine which peptide contains the major tyrosine phosphorylation site, we digested the tryptic phosphopeptide from TrpE-GAPp with chymotrypsin, thermolysin, or v8 protease and analyzed the products by two-dimensional mapping. This secondary digestion revealed that tryptic phosphopeptide a from TrpE-GAPp is resistant to chymotrypsin but sensitive to thermolysin and v8 protease (Fig. 6, F–H, and data not shown). This result suggests that tryptic phosphopeptide a from both full-length GAP and TrpE-GAPp consists of amino acids 458–464 of human GAP (EIYNTIR), which contains Tyr-460 as a phosphorylation site. Taken together, these results suggest that Tyr-460 of human GAP (corresponding to Tyr-457 of bovine GAP) is the major site of phosphorylation by Src.

Src Phosphorylates Tyr-460 of Human GAP Corresponding to Tyr-457 of Bovine GAP—To confirm our prediction, TrpE-GAPp[phe460] (containing Phe in place of Tyr-460) was expressed in bacteria and mixed with baculovirus-expressed v-Src as described above. Anti-Src mAb 2–17 immunoprecipitates were prepared and subjected to an \textit{in vitro} kinase assay with \[^{32}P\]ATP, and then TrpE-fusion proteins were immunopurified using anti-TrpE antibodies. Results indicate that phosphorylation of TrpE-GAPp[phe460] by v-Src was severely reduced compared with that of TrpE-GAPp (Fig. 7A). The tryptic peptide map of weakly phosphorylated TrpE-GAPp[phe460] by v-Src was severely reduced (not shown), suggesting that both c-Src and v-Src phosphorylate the same tyrosine residue in GAP.

To further confirm that Tyr-460 of human GAP is the major site of tyrosine phosphorylation by Src, we used a synthetic peptide with the sequence EIYNTIR. This peptide corresponds to the predicted tryptic peptide containing Tyr-460 of human GAP and Tyr-457 of bovine GAP (the sequence of this peptide is conserved in human and bovine GAP) (34). This synthetic peptide was phosphorylated \textit{in vitro} by the baculovirus-expressed v-Src and then purified from a TLC plate. When the purified phosphorylated synthetic peptide was mixed and analyzed simultaneously with tryptic phosphopeptide from TrpE-GAPp, the phosphorylated synthetic peptide comigrated with phosphopeptide from TrpE-GAPp (Fig. 7, B–D). Furthermore, the secondary digestion patterns of phosphorylated synthetic peptide by thermolysin and chymotrypsin were identical to those of phosphopeptide from TrpE-GAPp (Fig. 7, E–G). Together, these results demonstrate that the Src tyrosine kinases phosphorylate Tyr-460 of human GAP (Tyr-457 of bovine GAP) \textit{in vitro} as well as \textit{in vivo}.

**DISCUSSION**

We reported previously (16, 17) that GAP is associated in complexes with, and phosphorylated by, the Src kinases in immunoprecipitates prepared from vertebrate fibroblasts. Furthermore, these interactions between Src and GAP could
be reconstituted in an in vitro system using recombinant baculovirus-expressed proteins (18). In this work, we examined the specific sites of tyrosine phosphorylation of GAP in vitro as well as in vivo. Tryptic peptide mapping analyses of GAP from in vitro kinase reactions indicated that the major site of phosphorylation is Tyr-457 in bovine GAP (corresponding to Tyr-460 in human GAP). This residue of GAP was phosphorylated in kinase reactions using Src-GAP complexes that were either immunoprecipitated from rat fibroblasts or reconstituted from baculovirus-expressed proteins, suggesting that the reconstituted complexes are similar to endogenous complexes present in rat cell lysates. Moreover, tryptic peptide mapping of GAP metabolically labeled in intact rat fibroblasts transformed by v-Src revealed that the major site of tyrosine phosphorylation in vivo is the same as the major site of GAP phosphorylation by v-Src in vitro. Combined with the observation that GAP and Src associate in complexes (16–18), these results provide strong evidence that GAP is a direct substrate of the v-Src kinase in vivo. In addition, baculovirus-expressed c-Src kinase, which is an activated kinase in insect cells (33), phosphorylated the same tyrosine residue of GAP in vitro. This raises the possibility that the c-Src kinase, perhaps transiently activated in response to appropriate physiologic stimuli, could directly phosphorylate GAP in normal vertebrate cells.

In addition to the major site of tyrosine phosphorylation, other phosphorylation sites were detected in GAP. A minor phosphopeptide (phosphopeptide b) was observed in GAP phosphorylated in vitro by the Src kinases but was not detected in metabolically labeled rat fibroblasts. It is possible that this minor site of tyrosine phosphorylation is unique to in vitro kinase reactions or that phosphate at this site is more susceptible to phosphatases during lysis of metabolically labeled rat cells. Another minor site of tyrosine phosphorylation (phosphopeptide C) was detected in vivo but not in vitro, suggesting that a tyrosine kinase distinct from Src phosphorylates GAP at this site in intact cells. Identification of these minor sites of tyrosine phosphorylation of GAP in vitro and in vivo is in progress. Phosphoamino acid analysis of GAP from metabolically labeled rat fibroblasts further revealed that GAP contains substantial amounts of phosphoserine but very little phosphothreonine. This phosphoserine appears to be distributed among many sites in rat fibroblast GAP, because tryptic peptide mapping failed to identify prominent sites of serine phosphorylation.

We showed previously (18) that an amino-terminal portion of GAP containing SH2 regions contributes to complex formation with the Src kinase. Consistent with this finding, the SH2 regions of GAP expressed in bacteria as TrpE-fusion proteins were sufficient to associate with baculovirus-expressed Src in the present study. Furthermore, treatment of baculovirus-expressed v-Src with potato acid phosphatase prior to reconstitution of complexes significantly reduced phosphorylation of Src and its association with GAP. These results suggest that the SH2 regions of GAP interact with phosphorylated residues in Src. On the other hand, phosphorylation of Tyr-457 in GAP may not contribute to its association with Src, because TrpE-GAP fusion proteins that lack the corresponding residue of human GAP associate with Src in the in vitro reconstitution system. It remains possible that phosphorylation of this site in GAP might mediate interactions with other cellular proteins. Consistent with this possibility, tyrosine-phosphorylated GAP forms heteromeric complexes with at least two additional phosphoproteins, p62 and p190, in cells transformed by v-Src (7, 23, 24). Because TrpE-GAP fusion proteins lacking the major site of tyrosine phosphorylation in GAP directly associate with p62 from v-Src transformed cells (35), p190 is a better candidate than p62 for interacting with phosphotyrosine in GAP.

Previous studies (25) demonstrated that Tyr-460 of human GAP is the major site phosphorylated by the epidermal growth factor receptor kinase. Taken together with our findings that the equivalent residues of rat and bovine GAP are phosphorylated by Src kinases, these results suggest that specific phosphorylation at this site by different tyrosine kinases has physiologic significance. Earlier studies indicated that cellular Ras has an essential function in mitogenic signaling downstream of growth factor receptors and oncproteins with tyrosine kinase activity (11, 12). Because GAP is a regulator and possibly an effector of Ras function (14, 15), the finding that GAP is phosphorylated by diverse tyrosine kinases provides a biochemical bridge linking these kinases with Ras signaling pathways (7, 17). One possibility is that phosphorylation of Tyr-457 or the equivalent residue in GAP inhibits its GTPase-enhancing activity toward Ras, thereby preferentially keeping Ras in the activated GTP-bound state. Alternatively, regulation of GAP activity could occur indirectly through formation of heteromeric complexes with another cellular protein, such as p190 (24), whose association may be dependent on Tyr-457 phosphorylation.

Our results presented here, combined with results of other

\[\text{S. Park, X. Liu, T. Pawson, and R. Jove, unpublished results.}\]
studies described above, suggest a model for Src-induced oncogenesis wherein Tyr-457 or the equivalent residue of GAP phosphorylation by Src could contribute to subversion of normal growth control mechanisms. Although phosphorylation of this site by growth factor receptor kinases is tightly regulated in normal cells, constitutive phosphorylation of GAP by v-Src could stimulate uncontrolled cell proliferation. It will be of interest to determine whether Tyr-457 phosphorylation using baculovirus-expressed proteins should facilitate further growth control mechanisms. Although phosphorylation studies described above, suggest a model for Src-induced signaling, this site by growth factor receptor kinases is tightly regulated in normal cells, constitutive phosphorylation of GAP by v-Src could stimulate uncontrolled cell proliferation. It will be of interest to determine whether Tyr-457 phosphorylation using baculovirus-expressed proteins should facilitate further growth control mechanisms.

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