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The Epidermal Growth Factor Receptor Phosphorylates GTPase-Activating Protein (GAP) at Tyr-460, Adjacent to the GAP SH2 Domains

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GTPase-activating protein (GAP) stimulates the ability of $p21^{ras}$ to hydrolyze GTP to GDP. Since GAP is phosphorylated by a variety of activated or oncogenic protein-tyrosine kinases, it may couple tyrosine kinases to the Ras signaling pathway. The epidermal growth factor (EGF) receptor cytoplasmic domain phosphorylated human GAP in vitro within a single tryptic phosphopeptide. The same GAP peptide was also apparently phosphorylated on tyrosine in EGF-stimulated rat fibroblasts. Circumstantial evidence suggested that residue 460 might be the site of GAP tyrosine phosphorylation. This possibility was confirmed by phosphorylation of a synthetic peptide corresponding to the predicted tryptic peptide containing Tyr-460. Alteration of Tyr-460 to phenylalanine by site-directed mutagenesis diminished the in vitro phosphorylation of a bacterial GAP polypeptide by the EGF receptor. We conclude that Tyr-460 is a site of GAP tyrosine phosphorylation by the EGF receptor in vitro and likely in vivo. GAP Tyr-460 is located immediately C terminal to the second GAP SH2 domain, suggesting that its phosphorylation might have a role in regulating protein-protein interactions.

The biological properties of transmembrane and cytoplasmic protein-tyrosine kinases depend on the kinase activities of their catalytic domains. Growth factors such as epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) are unable to induce DNA synthesis when the tyrosine kinase activities of their receptors have been abolished by the substitution of residues which are critical for catalysis (reviewed in reference 27). Similarly, mutations which impair the tyrosine kinase activities of the v-*src* or v-*fps* oncogene product also abrogate their transforming activity (6, 25, 30). These results imply that the phenotypic changes induced by activated growth factors or oncogenic tyrosine kinases are mediated by tyrosine phosphorylation.

Activated growth factor receptors physically associate with, and phosphorylate, a number of cytoplasmic proteins that regulate intracellular signal transduction pathways. These include phospholipase C-γ (PLC-γ) (16, 18), $p60^{c\text{-}src}$ and Src-related proteins (12), phosphatidylinositol 3′-kinase (8, 9), $p74^{raf}$ (21), and Ras GTPase-activating protein (GAP) (7, 10). Although these proteins are enzymatically unrelated, several of them contain conserved noncatalytic SH2 domains (23) that apparently mediate their interactions with activated receptors (2, 20). Among these receptor-binding proteins, GAP is of considerable interest, as it is a strong candidate for the molecule that couples tyrosine kinases to $p21^{ras}$ (5, 19). GAP interacts with $p21^{ras}$ in the biologically active GTP-bound conformation and greatly stimulates its ability to hydrolyze GTP to GDP (25a, 28), thereby returning $p21^{ras}$ to the inactive GDP-bound form. GAP is therefore a negative regulator of $p21^{ras}$. Since GAP interacts with the effector region of GTP-bound $p21^{ras}$, it is possible that GAP or a GAP-associated protein functions as a Ras target (1, 3, 17).

GAP becomes phosphorylated on tyrosine in fibroblasts stimulated with EGF or PDGF and in cells transformed by tyrosine kinase oncogenes such as v-*src* (5, 7, 19). Activated tyrosine kinases also induce GAP to form multiple heteromeric complexes with other tyrosine-phosphorylated proteins, notably polypeptides of 62 and 190 kDa (p62 and p190) (5). GAP phosphorylation on serine and threonine is also increased in v-*src*-transformed cells (5).

To investigate the effects of tyrosine phosphorylation on presumptive tyrosine kinase targets such as GAP, it is important to identify the phosphorylated residue(s). Here we have localized a major site of GAP tyrosine phosphorylation to tyrosine 460. The position of this residue relative to the regulatory and catalytic domains of GAP has interesting implications for the possible function of GAP tyrosine phosphorylation.

MATERIALS AND METHODS

Construction of the fusion proteins TrpE-GAP_p and TrpE-GAPp[phe^{460}]. A *Bal*I-*Bgl*II fragment, encoding residues 171 to 520 of human GAP (26), was inserted between the *Eco*RI and *Bam*HI sites in the polylinker of the pATH3 *trpE* expression vector, giving a TrpE-GAP fusion protein of about 80 kDa. The *Eco*RI site was blunt ended prior to ligation.

To generate TrpE-GAPp[phe^{460}], a *Hin*dIII fragment of plasmid TrpE-GAPp containing the codon for Tyr-460 was subcloned into plasmid pTZ18R. Site-specific mutagenesis was performed by the uracil-substitution method, using the oligonucleotide AGGAAATCTTTAATACCATC, which changed codon 460 (TAT coding for Tyr) to TTT (Phe). The Phe-substituted *Hin*dIII fragment was subcloned back into TrpE-GAPp.

In vitro and in vivo phosphorylation of GAP, TrpE-GAP fusion proteins, and the synthetic peptide, $peptide_{460}$. R1hER cells, which are rat fibroblasts overexpressing the human EGF receptor (EGF-R), were seeded in 100-mm dishes and

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grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS) to confluency. The cells were serum starved overnight in DMEM with 0.5% FBS. Starved cells were rinsed once in labeling medium (DMEM minus phosphate, 0.5% FBS). Cells were labeled in 2 ml of labeling medium with 5 mCi of $^{32}P_i$ (285 Ci/mg of P; ICN) per dish for 2 h at 37°C. Sodium orthovanadate (Na_3VO_4) was added to a final concentration of 50 μM, and incubation continued for 30 min. EGF was then added directly to the labeling medium to a final concentration of 80 nM for 3 min at 37°C. Cells were lysed in lysis buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 10 μg of aprotinin per ml, 10 μg of leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride, 200 μM sodium orthovanadate, 10 mM tetrasodium pyrophosphate, 100 mM sodium fluoride). The clarified lysate was precleared by incubation at 4°C for 30 min with nonspecific rabbit immunoglobulin G immobilized on protein A-Sepharose. The precleared lysate was then incubated with affinity-purified anti-GAP antibodies and protein A-Sepharose at 4°C for 60 min. The immunoprecipitates were washed three times in the same buffer.

Purified baculovirus-expressed GAP (a gift of P. Polakis and F. McCormick) was incubated with purified baculovirus-expressed EGF-R cytoplasmic domain (a gift of B. Margolis and J. Schlessinger) in 50 μl of kinase reaction buffer (20 mM HEPES [pH 7.5], 25 mM $MgCl_2$, 4 mM $MnCl_2$, 0.1 mM Na_3VO_4). The in vitro kinase reaction was initiated by addition of 20 μM ATP and 100 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and was incubated for 30 min at room temperature. The reaction mixture was then diluted to 500 μl with cell lysis buffer (above) and immunoprecipitated with affinity-purified anti-GAP antibodies.

A pellet from 10 ml of *Escherichia coli* expressing TrpE fusion proteins was lysed by resuspension in 1 ml of bacterial lysis buffer (50 mM Tris-HCl [pH 7.5], 0.3 M NaCl, 1% Nonidet P-40, 20 mM $MnCl_2$) and then sonicated. Polyethylene glycol (PEG 3500; Sigma) was added to the clarified lysate to a final concentration of 9%, and the mixture was incubated on ice for 30 min. Precipitates were collected by centrifugation at 12,000 × *g* for 5 min, resuspended in 2 ml of 20 mM HEPES (pH 7.5)–10 mM $MnCl_2$, and washed twice in the same buffer by centrifugation in an Amicon microconcentrator (30-kDa cutoff). A fraction of the concentrated, clarified preparation was mixed with purified EGF-R cytoplasmic domain in 50 μl of kinase reaction buffer. Following the in vitro kinase reaction, the mixture was heated at 100°C for 3 min following the addition of 1% sodium dodecyl sulfate (SDS) and 10 mM dithiothreitol. The sample was diluted 10 times with the lysis buffer, and the phosphorylated fusion proteins were immunoprecipitated with affinity-purified anti-TrpE antibodies.

The synthetic peptide was dissolved in water (10 mg/ml), and 1 μl was added to 20 μl of kinase reaction buffer containing 3 μg of the EGF-R cytoplasmic domain previously immobilized on protein A-Sepharose beads, using an anti-EGF-R antibody. Kinase reactions were performed as described above. The supernatant, which contained phosphorylated peptide, was spotted directly on a thin-layer cellulose (TLC) plate for analysis.

Phosphoamino acid analysis, peptide cleavage, and peptide mapping. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon (for phosphoamino acid analysis) or nitrocellulose (for peptide mapping) membranes. Phosphorylated proteins were localized by autoradiography and excised. Proteins immobilized on Immobilon were hydrolyzed in 5.7 M HCl at 110°C for 1 h. The hydrolysates were dried in vacuum and analyzed by two-dimensional electrophoresis on TLC plates (4). For peptide mapping, immobilized proteins were digested directly on nitrocellulose membranes with either trypsin or CNBr. Trypsin (20 μg) digestion was carried out in 50 mM NH_4HCO_3 (200 μl) at 37°C overnight. CNBr (2 mg) digestion was carried out in 70% formic acid (200 μl) at room temperature overnight. Following digestion, the supernatants were dried under vacuum and washed two or three times with water. CNBr-digested peptides were separated on a 15% SDS-polyacrylamide gel and visualized by autoradiography. Tryptic peptides were oxidized with performic acid and analyzed by electrophoresis at pH 2.1 (first dimension) and chromatography (second dimension) on a cellulose-coated TLC plate (13). The phosphate-containing peptides were scraped from the TLC plate and eluted with 5% formic acid. Digestion of the eluted tryptic peptide with both *Staphylococcus aureus* V8 protease and chymotrypsin was carried out in 50 mM NH_4HCO_3 at 37°C overnight.

RESULTS

EGF induces GAP to become phosphorylated at multiple sites in vivo. Rat-1 fibroblasts overexpressing the human EGF-R (R1hER cells) were metabolically labeled with $^{32}P_i$, serum starved, pretreated with sodium orthovanadate for 30 min, and then stimulated with 80 nM EGF for 3 min. Affinity-purified anti-GAP antibodies specifically immunoprecipitated phosphorylated GAP, and the two GAP-associated proteins p62 and p190, from lysates of EGF-stimulated cells (Fig. 1A). p62 and p190 from EGF-stimulated cells had tryptic phosphopeptide maps which were indistinguishable from those of the corresponding GAP-associated proteins from v-*src*-transformed rat fibroblasts, suggesting that the same or very closely related proteins associate with GAP in response to the EGF-R or $p60^{v\text{-}src}$ tyrosine kinases. Identities of the other proteins that were also precipitated with anti-GAP antibodies (Fig. 1A, lane 1) are unknown, although they may represent additional GAP-associated proteins. Phosphoamino acid analysis of GAP, p62, and p190 from EGF-stimulated cells gave results similar to those previously obtained for v-*src*-transformed cells (5). p62 contained predominantly phosphotyrosine (70%) as well as phosphoserine, whereas GAP and p190 contained lower amounts of phosphotyrosine (24 and 10%, respectively) and proportionately more phosphoserine (Fig. 1B). In contrast to EGF-stimulated cells, an anti-GAP immunoprecipitate from unstimulated R1hER cells contained no detectable ^{32}P-labeled GAP or p190, although a phosphorylated 62-kDa protein was detected. This may represent a basal level of GAP-associated phospho-p62. To investigate GAP phosphorylation in more detail, ^{32}P-labeled GAP from EGF-stimulated cells was isolated, digested with trypsin, and analyzed by two-dimensional mapping, using electrophoresis and chromatography on TLC plates (Fig. 1C). GAP contained four major tryptic phosphopeptides (a to d), of which only peptide c yielded phosphotyrosine when the isolated peptides were subjected to phosphoamino acid analysis (data not shown). A very similar tryptic phosphopeptide map was obtained with GAP isolated from ^{32}P-labeled v-*src*-transformed rat cells (data not shown). These results suggested that GAP is modified at a single tyrosine, or possibly at multiple closely spaced tyrosine residues, by the EGF-R in vivo. As described in Materials and Methods, we used sodium orthovanadate to

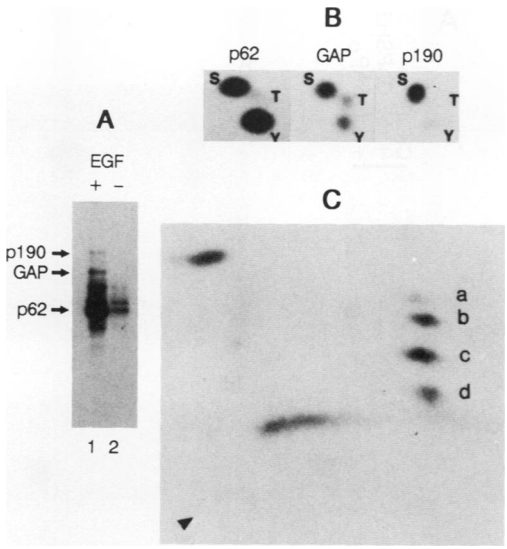

FIG. 1. Tyrosine phosphorylation of GAP induced by EGF stimulation. (A) R1hER cells were labeled with $^{32}P_i$ for 2.5 h, with the addition of 50 μM Na_3VO_4 for the last 30 min. ^{32}P-labeled cells were either stimulated with 80 nM EGF for 3 min at 37°C (lane 1) or unstimulated (lane 2). Cell lysates were immunoprecipitated with affinity-purified anti-GAP antibodies, and the immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel and visualized by autoradiography. Phosphorylated GAP and the two GAP-associated proteins, p62 and p190, are indicated by arrows. (B) Phosphorylated p62, GAP, and p190 were hydrolyzed and subjected to phosphoamino acid analysis by two-dimensional electrophoresis at pH 1.9 and 3.5, respectively. The positions of the three unlabeled phosphoamino acid markers are indicated by Y (phosphotyrosine), T (phosphothreonine), and S (phosphoserine). (C) GAP tryptic phosphopeptides were analyzed on a TLC plate by electrophoresis at pH 2.1 (horizontal, anode to the left) and ascending chromatography (vertical). The four major phosphotryptic peptides (a through d) and the origin (arrowhead) are indicated.

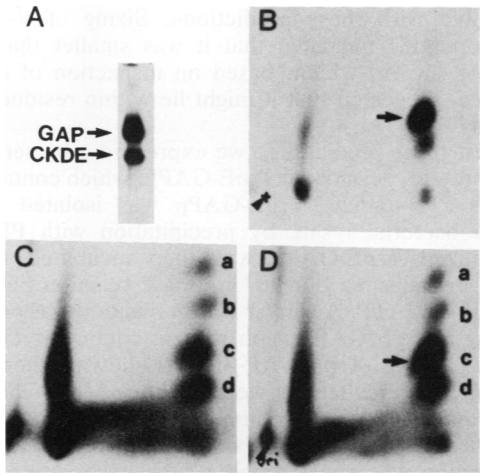

FIG. 2. Comparative tryptic phosphopeptide mapping of GAP phosphorylated by the EGF-R in vivo and in vitro. (A) A 1-μg sample of GAP was incubated with 1 μg of the EGF-R cytoplasmic domain (CKDE) in a total volume of 50 μl of kinase reaction buffer (Materials and Methods) with 100 μCi of [γ-^{32}P]ATP. After incubation for 30 min at room temperature, the mixture was diluted to 500 μl with cell lysis buffer and immunoprecipitated with anti-GAP antibodies. The immunoprecipitate was analyzed by SDS-PAGE and autoradiography. ^{32}P-labeled GAP and the EGF-R kinase polypeptide are indicated. (B) Two-dimensional tryptic phosphopeptide map of in vitro-phosphorylated GAP from panel A (300 cpm, 4 days of exposure). The single phosphopeptide is indicated by an arrow. The other minor spots were not detected in several other similar experiments (see Fig. 3 for example) and are thus believed to be partial digest products. The arrowhead marks the origin. (C) Tryptic phosphopeptide map of in vivo-phosphorylated GAP (1,200 cpm, 4 days of exposure). Peptide designations (a to d) are the same as in Fig. 1C. (D) Tryptic phosphopeptides of GAP phosphorylated in vivo (1,200 cpm) and in vitro (300 cpm) were mixed prior to two-dimensional separation (4 days of exposure).

increase the incorporation of phosphate into GAP tyrosine residues, without which we were unable to obtain sufficient ^{32}P in phosphopeptide c for subsequent phosphoamino acid analysis. Although there was no detectable tyrosine phosphorylation of GAP in the absence of EGF stimulation, we cannot exclude the possibility that the use of vanadate induces the tyrosine phosphorylation of a nonphysiological site.

The EGF-R kinase phosphorylates GAP within the same tryptic peptide in vitro and in vivo. To confirm that GAP is a direct substrate for the EGF-R tyrosine kinase, we studied GAP phosphorylation in vitro by the EGF-R cytoplasmic domain. Full-length human GAP was incubated with a polypeptide containing the cytoplasmic kinase domain of the EGF-R (CKDE), both purified from baculovirus expression systems (7, 22). GAP was phosphorylated in vitro by the EGF-R cytoplasmic domain (Fig. 2A). Furthermore, both GAP and the EGF-R polypeptide were present in an anti-GAP immunoprecipitate obtained from the in vitro kinase reaction, indicating a physical association of the kinase and the substrate (Fig. 2A). Phosphoamino acid analysis of GAP phosphorylated in vitro by the EGF-R cytoplasmic domain confirmed that phosphorylation occurred exclusively on tyrosine residues (data not shown). Digestion of in vitro-phosphorylated GAP with trypsin generated a single phosphopeptide (Fig. 2B). Thus, as with the in vivo response to EGF, GAP seems to be phosphorylated on a single tryptic peptide by the purified EGF-R kinase domain in vitro.

When the tryptic phosphopeptides derived from both in vivo- and in vitro-phosphorylated GAP were mixed and analyzed simultaneously, it was apparent that the single phosphopeptide derived from in vitro-phosphorylated GAP comigrated with peptide c from in vivo-phosphorylated GAP (Fig. 2D). This is consistent with the observation that peptide c is the only phosphotyrosine-containing tryptic peptide isolated from in vivo-phosphorylated GAP. We conclude that the EGF-R cytoplasmic domain very probably phosphorylates GAP in vitro on the same tyrosine residue as does the activated EGF-R in intact cells.

The site of in vitro GAP tyrosine phosphorylation is located between residues 448 and 520 in human GAP. There are 26 potential tryptic peptides in human GAP which contain at least one tyrosine. In an effort to localize the modified tyrosine residue within a specific region of GAP, we digested the in vitro-phosphorylated GAP with CNBr. CNBr digestion yielded a major phosphopeptide of about 10 kDa. A larger phosphopeptide of about 24 kDa was also detectable (data not shown), possibly representing a partial cleavage product. This pattern suggested that the phosphorylated tyrosine residue is located in a relatively large CNBr fragment of approximately 10 kDa, which may be flanked by another large peptide in order to generate the 24-kDa partial CNBr fragment. Sequences 3 to 224 and 444 to 673 are both

compatible with these predictions. Sizing of the tryptic phosphopeptide indicated that it was smaller than 2 kDa (data not shown) which, based on inspection of the GAP sequence, suggested that it might lie within residues 201 to 224 or 444 to 483.

To test these predictions, we expressed a bacterial TrpE fusion protein, designated TrpE-GAPp, which contains GAP residues 171 to 520. TrpE-GAPp was isolated from an induced bacterial lysate by precipitation with PEG. The resolubilized TrpE-GAPp was then incubated with the EGF-R cytoplasmic domain in kinase reaction buffer containing [γ-^{32}P]ATP. A similar kinase reaction was performed by using a TrpE-GAP fusion protein containing GAP residues 171 to 448 (TrpE-GAP-SH2). Following these kinase reactions, the incubations were denatured by boiling in 1% SDS, diluted, and then immunoprecipitated with anti-TrpE antibodies to recover the bacterial fusion proteins. The denaturation was necessitated by the observation that the TrpE-GAPp fusion protein comigrates with the autophosphorylated CKDE EGF-R polypeptide, which tends to associate with and obscure the bacterial fusion protein. The EGF-R cytoplasmic domain phosphorylated TrpE-GAPp but not TrpE-GAP-SH2 (Fig. 3A), even though equivalent amounts of the two fusion proteins were present, as determined by immunoblotting with anti-TrpE antibodies (data not shown, but see Fig. 5). Tryptic phosphopeptide mapping of the ^{32}P-labeled 80-kDa TrpE-GAPp band revealed one major and several minor phosphopeptides (Fig. 3). The major tryptic phosphopeptide of TrpE-GAPp comigrated with the tryptic phosphopeptide obtained from full-length GAP phosphorylated in vitro by the EGF-R kinase (Fig. 3). This finding suggests that TrpE-GAPp is phosphorylated by the EGF-R kinase at a physiological tyrosine phosphorylation site. The minor phosphopeptides from the TrpE-GAPp band comigrated with tryptic phosphopeptides derived from autophosphorylated CKDE EGF-R (data not shown) and hence represent residual CKDE protein in the immunoprecipitate. The weakly labeled 80-kDa band from kinase reactions lacking bacterial fusion protein or containing TrpE-GAP-SH2 (Fig. 3A, lanes 1 and 2) was autophosphorylated CKDE EGF-R, as judged by tryptic phosphopeptide analysis (data not shown). The more rapidly migrating ^{32}P-labeled band (Fig. 3A, lanes 2 and 3) gave tryptic phosphopeptides which did not comigrate with those from GAP or CKDE, and its identity is therefore uncertain. The phosphorylation of TrpE-GAPp but not TrpE-GAP-SH2 by the EGF-R kinase suggests that the major GAP tyrosine phosphorylation site is located between GAP residues 448 and 520. A soluble bacterial v-*abl* tyrosine kinase phosphorylated TrpE-GAPp on the same tryptic peptide as did the EGF-R kinase (data not shown).

Tyr-460 in human GAP is phosphorylated in vitro by the EGF-R. After roughly localizing the phosphotyrosine residue in GAP, we performed secondary proteolytic digestion of the tryptic phosphopeptide derived from in vitro-phosphorylated GAP. The phosphotyrosine-containing tryptic peptide was resistant to chymotrypsin (data not shown) but was susceptible to *S. aureus* V8 protease (Fig. 4). There are two predicted tyrosine-containing tryptic peptides (LLYP VAPPEPVEDR and EIYNTIR, containing Tyr-270 and Tyr-460, respectively) between residues 171 and 520 which are compatible with this profile of protease sensitivity. We eliminated the former, since TrpE-GAP-SH2, which contains this site, was not appropriately phosphorylated by the EGF-R kinase domain in vitro (see above). To test whether Tyr-460 might be the site of tyrosine phosphorylation, we

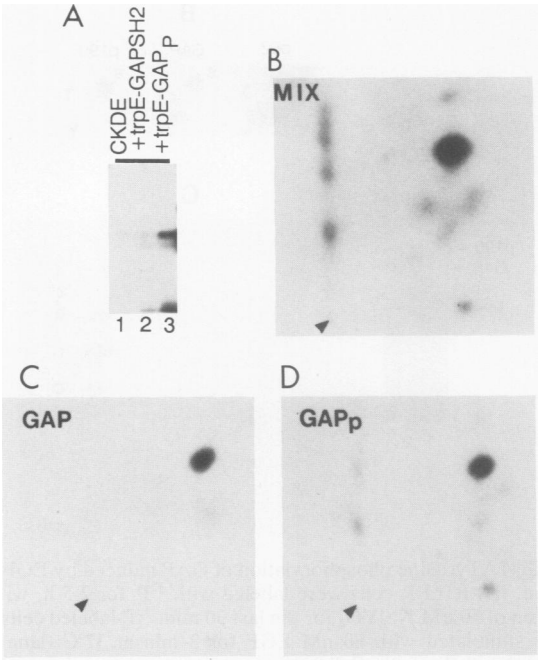

FIG. 3. Evidence that bacterial fusion protein TrpE-GAPp is phosphorylated in vitro on the same tyrosine residue as intact GAP. (A) A 1-μg sample of purified EGF-R cytoplasmic domain (CKDE) was incubated either alone (lane 1) or with PEG-precipitated TrpE-GAP-SH2 (lane 2) or TrpE-GAPp (lane 3) in 50 μl of kinase reaction buffer containing 50 μCi of [γ-^{32}P]ATP for 30 min at room temperature. The amount of the respective bacterial TrpE fusion protein in each reaction was 2 μg. Kinase reactions were boiled in SDS, diluted, and immunoprecipitated with anti-TrpE antibodies. The immunoprecipitates were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Phosphorylated proteins were visualized by autoradiography. The arrow indicates the position of phosphorylated TrpE-GAPp. (B to D) Full-length GAP (Fig. 2A) or phosphorylated TrpE-GAPp (above) was digested with trypsin. The resulting peptides (400 cpm in each case) were subjected to two-dimensional peptide mapping either alone (C and D) or together (B). All autoradiographs were exposed for 2 days at −70°C.

obtained a synthetic peptide, peptide$_{460}$, with the sequence EIYNTIR, which corresponds to the predicted tryptic peptide containing Tyr-460 of human GAP. Peptide$_{460}$ was readily phosphorylated in vitro by the EGF-R polypeptide. Peptide$_{460}$ which had been phosphorylated by the EGF-R cytoplasmic domain comigrated with the tryptic phosphopeptide obtained from in vitro-phosphorylated human GAP (Fig. 4). Furthermore, the V8 protease digestion pattern of phosphorylated peptide$_{460}$ was identical to that of the V8 protease product of the tryptic phosphopeptide derived from full-length GAP. By mixing samples A and D (Fig. 4), we have determined that the more slowly ascending peptide seen following V8 protease digestion (Fig. 4D) represents the original tryptic phosphopeptide, while the more rapidly ascending peptide is the V8 protease-digested product (data not shown). This result is consistent with the prediction that removal of the single N-terminal glutamate from peptide$_{460}$ as a consequence of V8 protease digestion would leave the peptide less negatively charged and more hydrophobic. Thus, we conclude that GAP Tyr-460 is phosphorylated by the purified EGF-R kinase domain in vitro and that the same residue is probably phosphorylated following activation of the EGF-R tyrosine kinase in vivo.

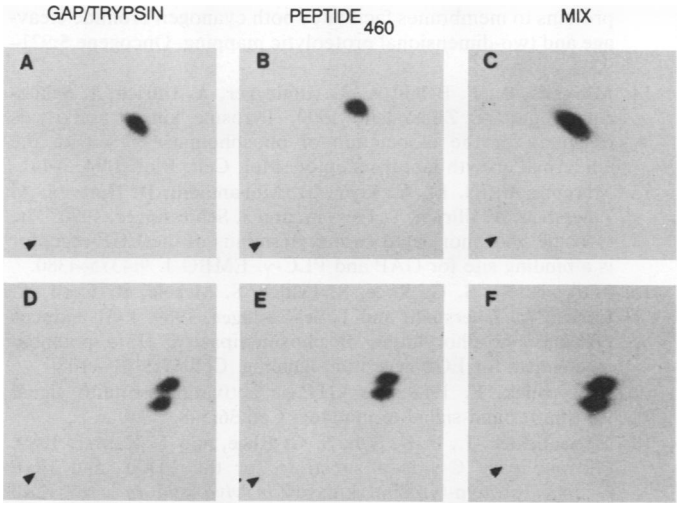

FIG. 4. Evidence that phosphorylated peptide$_{460}$ is indistinguishable from the tryptic phosphopeptide derived from in vitro-phosphorylated GAP. The phosphopeptides derived by trypsin digestion of GAP which had been phosphorylated in vitro by the EGF-R cytoplasmic domain (CKDE) and peptide$_{460}$ phosphorylated by CKDE were purified from TLC plates following two-dimensional separation. The purified phosphopeptides were reanalyzed on TLC plates in two dimensions either alone (A [GAP] and B [peptide$_{460}$]) or together (C). The purified phosphopeptides were further digested with *S. aureus* V8 protease (D, E, and F), and the resulting peptides were analyzed either alone (D [GAP] and E [peptide$_{460}$]) or together (F). In all cases, the single sample had 150 cpm and the mixture had 150 cpm of each component. The autoradiographs were all exposed for 2 days at −70°C with an intensifying screen. The arrowheads point to the origin.

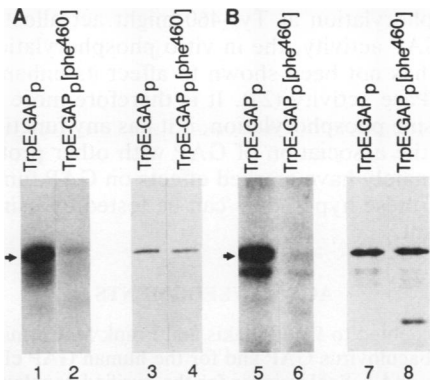

FIG. 5. Bacterial fusion protein TrpE-GAPp[phe^{460}] is poorly phosphorylated by either the EGF-R cytoplasmic kinase domain or p43$^{v\text{-}abl}$. A 1-μg sample of purified EGF-R cytoplasmic domain (CKDE; A) or 2 μl of p43$^{v\text{-}abl}$ (Oncogene Science; B) was incubated with 2 μg of either TrpE-GAPp or TrpE-GAPp[phe^{460}] (prepared as described in Materials and Methods) in 50 μl of kinase reaction buffer with 30 μCi of [γ-^{32}P]ATP for 30 min at room temperature. Samples in panel B (lanes 5 through 8) were analyzed directly. In panel A (lanes 1 through 4), the reaction mixtures were boiled in SDS and diluted, and the fusion proteins were precipitated with affinity-purified anti-GAP antibodies. Samples were split into two aliquots and separated by SDS-PAGE. One aliquot of each sample was analyzed by autoradiography (lanes 1, 2, 5, and 6), and the other aliquot was analyzed by Western immunoblotting, using mouse monoclonal anti-TrpE antibodies (Oncogene Science) and the alkaline phosphatase color reaction (lanes 3, 4, 7, and 8).

To test this possibility more rigorously, we mutated the codon for GAP Tyr-460 to phenylalanine and introduced this mutation into the TrpE-GAPp vector. Phosphorylation of TrpE-GAPp containing the Phe-460 substitution by either the EGF-R kinase or the v-*abl* kinase was greatly reduced (Fig. 5).

DISCUSSION

We have identified Tyr-460 of human GAP as a prominent site of in vitro phosphorylation by the EGF-R. Tyr-460 of human GAP (26) corresponds to Tyr-457 of bovine GAP (28). The tryptic phosphopeptide containing phosphorylated Tyr-460 of human GAP comigrated with the only phosphotyrosine-containing tryptic peptide obtained from rat GAP, which had been phosphorylated in R1hER cells in response to EGF stimulation. This result strongly suggests that Tyr-460, or its equivalent in rat GAP, is also the primary site of tyrosine phosphorylation in vivo. Tyr-460 is the only significant site of tyrosine phosphorylation that we have detected by analysis of tryptic phosphopeptides. This does not rule out the possibility that there are other sites of tyrosine phosphorylation, contained within tryptic phosphopeptides which are relatively insoluble and therefore lost during the peptide mapping procedure.

The location of Tyr-460 suggests some possible functions of GAP tyrosine phosphorylation. Tyr-460 is positioned immediately C terminal to the second GAP SH2 domain, in a position analogous to tyrosine phosphorylation sites in PLC-γ1. PLC-γ1 is phosphorylated on four tyrosine residues by the EGF-R in vitro, of which two (Tyr-771 and Tyr-783) are located just after the second PLC-γ1 SH2 domain (11). Of these PLC-γ1 sites, Tyr-771 is definitely phosphorylated in vivo in response to EGF stimulation, as is Tyr-1254 close to the C terminus (29). In this context, it is of interest that the form of PLC-γ1 that coprecipitates with the EGF-R from EGF-stimulated cells is poorly tyrosine phosphorylated (14). In vitro, PLC-γ1 complexed with the autophosphorylated EGF-R dissociates more rapidly following its phosphorylation by the receptor (15). Taken together, these results suggest that one function of PLC-γ1 tyrosine phosphorylation might be to decrease the strength of its interaction with the EGF-R (15).

The SH2 domains of both GAP and PLC-γ1, when expressed in isolation as bacterial fusion proteins, retain the capacity to bind autophosphorylated EGF and PDGF receptors (2, 20). These results indicate that SH2 domains mediate the physical association of GAP and PLC-γ1 with activated growth factor receptors. The N-terminal SH2 domain of GAP also complexes with tyrosine phosphorylated p62 in vitro, suggesting that this is the site of p62 binding in vivo (20). This common mechanism of receptor binding, involving SH2 domains, may explain why major sites of GAP and PLC-γ1 tyrosine phosphorylation are similarly located adjacent to the C-terminal SH2 domains. This might be expected if their SH2 domains orient GAP and PLC-γ1 in a similar fashion relative to the EGF-R tyrosine kinase active site. The location of GAP Tyr-460 and PLC-γ1 Tyr-771 or Tyr-783 next to the C-terminal SH2 domains is consistent with the possibility that their phosphorylation modulates the interactions of GAP or PLC-γ1 with autophosphorylated receptors or other phosphotyrosine-containing proteins.

Tyr-460 is some distance in the GAP primary structure from the C-terminal domain that interacts with p21^{ras} to stimulate its GTPase activity. Although it is quite feasible

that phosphorylation at Tyr-460 might act allosterically to regulate GAP activity, the in vitro phosphorylation of purified GAP has not been shown to affect its enhancement of $p21^{ras}$ GTPase activity (24). It is therefore more likely that GAP tyrosine phosphorylation, if it has any function, acts to modulate the association of GAP with other proteins. This might ultimately have marked effects on GAP function. The validity of these hypotheses can be tested by using Phe-460 GAP mutant.

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