Phosphorylation of the p190 RhoGAP N-terminal domain by c-Src results in a loss of GTP binding activity

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Abstract p190 RhoGAP is a multi-domain protein that is thought to regulate actin cytoskeleton dynamics. It can be phosphorylated both in vitro and in vivo at multiple sites by the Src tyrosine kinase and one or more of these sites is postulated to modulate p190 function. One of the regions which is multiply phosphorylated by Src in vitro is the N-terminal GTP binding domain. Using a partially purified, bacterially expressed recombinant protein that includes the GTP binding domain (residues 1–389), we show that GTP binds to this fragment in a specific and saturable manner that is both time- and dose-dependent and that tyrosine phosphorylation of this fragment by c-Src results in a loss of GTP binding activity. These findings suggest that tyrosine phosphorylation of the p190 N-terminal domain can alter its ability to bind GTP.

Key words: p190 RhoGAP; c-Src; GTP binding; Tyrosine; Phosphorylation

1. Introduction

p190 RhoGAP is a 190 kDa protein that was first identified as a tyrosine phosphorylated protein that co-immunoprecipitated with p120 RasGAP [1]. Molecular cloning of p190 cDNA revealed homologies in the N-terminal 250 amino acids with GTP binding proteins and in the C-terminal region with GTPase activating proteins for Rho family members (Fig. 1A [2,3]). GTP binding proteins and RhoGAPs have been associated with regulating actin dynamics, suggesting that p190 may also be involved in such activities [4]. Furthermore, tyrosine phosphorylation of p190 appears to provide a link between tyrosine kinase growth factor receptors, Ras signaling pathways (through RasGAP) and cytoskeleton remodeling. Studies in C3H10T1/2 murine fibroblasts showed that p190 RhoGAP and p120 RasGAP undergo transient, simultaneous re-distribution following epidermal growth factor stimulation and that this re-distribution correlates temporally and spatially with actin stress fiber dissolution [5]. Tyrosine phosphorylation of p190 by c-Src was also shown to co-ordinately regulate p190/p120 re-distribution and actin remodeling in an EGF-dependent manner. These findings led to the hypothesis that tyrosine phosphorylation could exert its effect on these processes through modulation of the GTP binding or RhoGAP activities of p190 N-terminal or C-terminal domains, respectively. The primary site of p190 tyrosine phosphorylation is Tyr 1105, which lies just upstream of the RhoGAP domain [6,7]. Phosphorylation of Tyr 1105 promotes p120 Ras GAP binding to p190 both in vitro and in vivo [6,7] and plays a role in vivo in modulating the activity of the p190 RhoGAP domain (Haskell et al., in preparation). However, other sites (as yet unidentified) are also phosphorylated in vivo and in vitro, particularly by v-Src [6], an oncoprotein that causes striking alterations in intracellular actin cytoskeleton structures [8]. Because there was precedence for tyrosine phosphorylation of GTP binding proteins having effects on their activities (see Section 4), we sought to determine if the ability of the isolated N-terminal domain to bind GTP in vitro might be affected by c-Src-mediated tyrosine phosphorylation. We found that tyrosine phosphorylation of the N-terminal domain by c-Src resulted in a decreased ability of this domain to bind GTP.

2. Materials and methods

2.1. Construction of p190 N-terminal bacterial expression plasmid

Full-length *wt* p190 cDNA (Fig. 1A) was excised from pBluescript (a gift of J. Settleman) by digestion with *Bam*HI and *Eco*RV and inserted into the pRSET A bacterial expression plasmid (Invitrogen, Carlsbad, CA, USA) at the compatible restriction sites, *Bam*HI and *Pvu*II. This construct (p190pRSET) encoded p190 that was hexahistidine tagged at the amino terminus. P190pRSET was then digested with *Nco*I, which resulted in the excision of the 3′ two-thirds of the p190 coding sequence and the formation of a fragment (retained in the plasmid backbone) that encoded the first 389 amino acids of p190. This fragment was isolated, self-ligated and transformed into the λDE3 lysogen of *Escherichia coli* BL21 for propagation and expression. A mutant form of this construct that was defective for GTP-binding was generated by substituting D for K201 and V for D203 (Fig. 1A), using the Quickchange mutagenesis kit from Stratagene. These residues are contained within the guanine nucleotide binding loop and the alteration of homologous residues in other GTP binding proteins has been shown to disrupt guanine nucleotide binding [9,10].

2.2. Expression and purification of p190 N-terminal domain

BL21/DE3 transformants were grown in LB media containing 50 μg/ml ampicillin at 37°C to an OD^{600} of approximately 1.0, at which point they were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG; Sigma) for 3 h. The bacteria were harvested by centrifugation and pellets were resuspended in chilled binding buffer (20 mM Tris–HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.01% NP-40, aprotinin, leupeptin and 1 mM PMSF). The suspension was pulse-sonicated on ice for 1 min to shear chromosomal DNA and cellular debris was pelleted by centrifugation at 10 000 rpm for 30 min in a Sorvall SS34 rotor. These and subsequent purification steps were carried out at 4°C. Supernatant, which contained the p190 N-terminal domain, was passed over a 2 ml bed volume Ni^{2+} charged resin (Novagen, Madison, WI, USA) at a flow rate of 20 ml/h. The column was then washed with 25 ml binding buffer, followed by a wash in 100 ml of binding buffer containing 60 mM imidazole. The protein bound to the Ni^{2+} resin was then eluted with 10 ml binding buffer containing

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1 M imidazole. One ml fractions were collected and analyzed by SDS–PAGE and Coomassie Blue staining or Western blotting to determine the fractions which contained the N-terminal domain. Protein concentrations of pooled fractions were determined by the bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL, USA). BL21/DE3 transformants of the pRSET vector without insert were grown and processed identically to serve as controls for GTP binding.

2.3. Western immunoblotting

All steps were performed at room temperature. Proteins separated by SDS–PAGE were transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA). The membrane was then incubated for 1 h in blocking buffer (4% bovine serum albumin (BSA) in Tris-buffered saline containing 0.1% Tween 20) and for an additional hour in blocking buffer containing 5 μg/ml purified, mouse monoclonal antibody (mAb) 8C10, which is specific for p190 [5,11]. To localize bound primary antibody, the membrane was incubated with ^{125}I-labeled goat anti-mouse IgG (New England Nuclear (NEN), Boxton, MA, USA) at a concentration of 1 μCi/ml in blocking buffer. The membrane was then washed three times for 5 min each in TBS with 0.1% Tween 20, air dried and subjected to autoradiography.

2.4. GTP overlay assay

This assay was carried out as described by Wagner et al. [12]. Briefly, 1–5 μg Ni^{2+}-column purified, His-tagged p190 N-terminal domain or equivalent control eluate from vector-alone extracts was separated by SDS–PAGE on a 7% gel. The gel was then incubated in 1 l 50 mM Tris–HCl, pH 7.5, 20% (v/v) glycerol for 30 min at room temperature. Using a Trans-Blot cell (Bio-Rad), proteins were transferred overnight at 150 mA, 4°C to nitrocellulose membranes, which were submerged in a buffer consisting of 10 mM $NaHCO_3$/3 mM Na_2CO_3, pH 9.8, to allow for protein renaturation. The membrane was then rinsed twice in 20 ml GTP binding buffer (50 mM NaH_2PO_4, pH 7.5, 10 μM $MgCl_2$, 2 mM DTT, 0.3% (v/v) Tween 20, 4 μM ATP) for 10 min each and incubated in 20 ml GTP binding buffer containing 40 μCi [α-^{32}P]GTP (3000 Ci/mmol, 10 mCi/ml; Dupont/NEN, Boston, MA, USA) for 2 h. The membrane was then washed six times for 2–5 min each in GTP binding buffer, dried and exposed to X-ray film.

2.5. GTP solution binding assay

The GTP solution binding assay was adapted from Tisdale et al. [13] and Graber et al. [14]. Approximately 1 pmol Ni^{2+}-column purified, His-tagged p190 N-terminal domain, an equivalent volume of a Ni^{2+}-column eluate from a vector-only control lysate or ~1 pmol of a partially purified preparation of the α subunit of the heterotrimeric G protein, G_{i3} (a gift of J. Garrison [14] were incubated with 1 μCi [γ-^{35}S]GTP (1250 Ci/mmol, Dupont/NEN, Boston, MA, USA, adjusted to 1 μCi/445 pmol with cold GTP) in a buffer containing 75 mM Na-HEPES, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM ATP and 1 mM EGTA (binding buffer) in a total volume of 200 μl for the indicated times at 30°C. The reaction was terminated by the addition of 1 ml chilled buffer containing 50 mM Tris–HCl, pH 8.0, 100 mM NaCl and 25 mM $MgCl_2$ (stop buffer) and placing the samples on ice. Samples were then filtered through 100 mm PVDF membrane discs (Millipore) and washed with 10 ml of a solution containing 25 mM Tris, 100 mM NaCl and 30 mM $MgCl_2$ (wash buffer). After air drying, the amount of labeled nucleotide bound to the filter was determined by scintillation counting. For dose dependency and competition assays, indicated concentrations of various nucleotides were added to the binding buffer containing [γ-^{35}S]GTP, and in competition studies, ATP was removed entirely from this buffer.

2.6. Src phosphorylation of p190 N-terminal domain

Prior to the kinase reaction, imidazole was removed from the preparation of p190 protein by either equilibrium dialysis in 50 mM Tris–HCl, pH 7.2, 150 mM NaCl or by gel filtration using a PD-10 column (Pharmacia). Approximately 1 μg Ni^{2+}-column purified p190 N-terminal domain was then incubated for 30 min in kinase reaction buffer (50 mM Tris–HCl, pH 7.2, 100 μM cold ATP and 10 mM $MgCl_2$) in the presence or absence of 3 units baculovirus-expressed c-Src (UBI). A soluble GTP binding assay was then carried out for 1 h with [γ-^{35}S]GTP, as described above, in the presence or absence of excess (10 μM) unlabeled GTP. To visualize phosphorylated proteins, ~1 μCi [γ-^{32}P]ATP was included in a separate in vitro kinase reaction and products were analyzed by SDS–PAGE and autoradiography.

2.7. Tryptic phosphopeptide mapping

Tryptic phosphopeptides of the in vitro phosphorylated p190 N-terminal domain were generated and analyzed according to the method of Boyle et al. [15]. Peptides were separated on 10×10 cm thin layer cellulose plates (E. Merck, Darmstadt, Germany) by electrophoresis in the first dimension at 1500 V for 22 min in pH 1.9 buffer (2.2% formic acid, 7.8% glacial acetic acid), using a Hunter thin-layer electrophoresis unit (HTLE 7000; CBS Scientific, Del Mark, CA, USA) and by chromatography in the second dimension in isobutyric acid buffer (isobutyric acid:*n*-butanol:pyridine:glacial acetic acid:H_2O at 62.5:1.9:4.8:2.9:27.9, v/v/v/v). Separated phosphotryptic peptides were visualized by autoradiography.

For determination of candidate sites phosphorylated by c-Src, the entire tryptic digest of radiolabeled p190 N-terminal domain was analyzed by C18 HPLC and isolated peptides were subjected to Edman degradation according to the method of Shannon and Fox [16].

3. Results

3.1. GTP binding to the p190 GTPase domain

To initiate our investigation into the possible regulation by tyrosine phosphorylation of the p190 N-terminal domain, we first wished to verify that the recombinant, purified protein was capable of binding GTP in a specific and saturable manner. To this end, BL23/DE3 *E. coli* bacteria were transformed with the pRSET expression vector encoding the His-tagged N-terminal fragment of p190 (Fig. 1A) and the recombinant protein was purified by Ni^{2+} chromatography as described in Section 2. The purity was assessed by Coomassie blue staining. As a negative control, bacteria were transformed with an empty vector, and the extract lacking recombinant p190 was carried through the same purification scheme as the extract with recombinant p190 protein. A mutant recombinant p190 N-terminal protein that contained K201D and D203V substitutions known to disrupt guanine nucleotide binding in other G proteins [9,10] was also generated and purified in the same manner (Fig. 1A). Fig. 1B is a Coomassie blue stain of the three preparations. Several contaminating bands were seen to variable extents in the different preparations of p190 (compare lanes 1 and 3 with lane 2), but the predominant product of the purification scheme migrated at ~50 kDa, the predicted size of the N-terminal recombinant p190 domain. The wild type protein preparation frequently contained several p190-specific breakdown products, as assessed by direct immunoblotting of the same panel of proteins with mAb 8C10, a p190-specific antibody [15] (Fig. 1C, lane

Table 1
P190 N-terminal domain preferentially binds GTP and GDP[a]

Competing nucleotide[b]	Relative binding of [γ-^{35}S]GTP[c]
–	1.00
GTPγS	0.43
GTP	0.34
dGTP	0.94
GDP	0.31
GMP	0.97
ATP	0.99
ADP	1.00
CTP	0.97
CDP	0.98
UTP	0.88

[a]Similar results were obtained in two additional experiments using 1000-fold molar excess unlabeled nucleotide.
[b]2500 pmol of unlabeled competing nucleotide: 5 pmol [γ^{35}S]GTP. (500-fold excess).
[c]Values represent the average of two independent experiments.

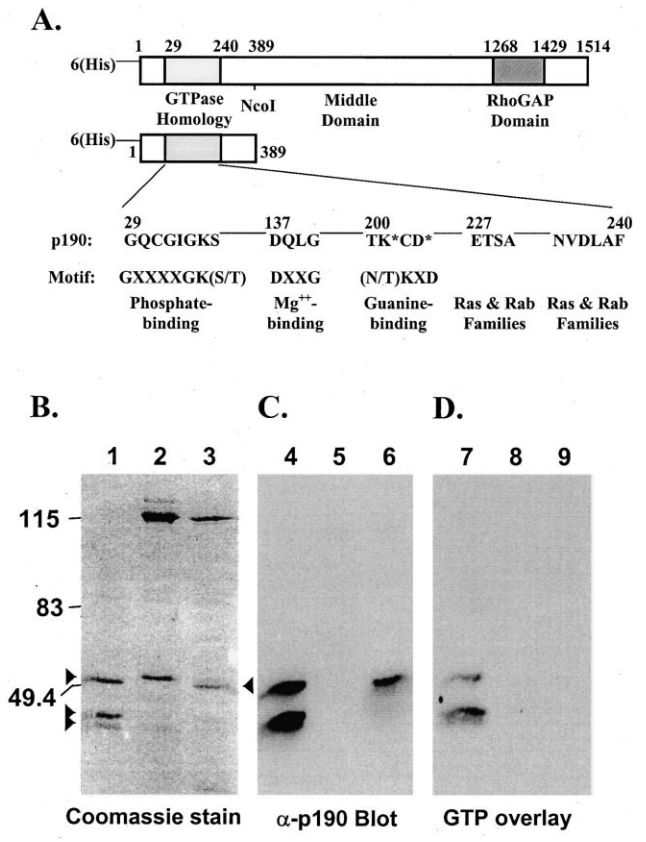

Fig. 1. Recombinant, hexahistidine-tagged p190 N-terminal domain binds GTP directly. A: Schematic of full-length p190 RhoGAP and the N-terminal GTP binding domain used in this study (residues 1–389). Full-length p190 contains an N-terminal GTP binding domain, a large middle domain and a C-terminal RhoGAP domain. The GTP binding domain, residues 29–240, contains three motifs found in all GTP binding proteins, a phosphate-binding loop, a Mg^{2+}-binding loop and a guanine nucleotide-binding loop. In addition, p190 contains sequences that contribute to the GTP binding capacity of Ras and Rab family members. The consensus motif for these regions, as well as the specific residues found in p190, are shown. Residues mutated in the guanine-binding loop are designated with *. B: Coomassie blue staining of an SDS–PAGE gel containing ~3 μg Ni^{2+} chromatography-purified *wt* p190 N-terminal domain (lane 1), vector only contaminating proteins (lane 2) and the K201D, D203V mutant of the N-terminal domain (lane 3). Arrows indicate the 50 kDa full-length, N-terminal fragment and two breakdown products. C: Western blot of the same samples depicted in (A), blotted with the anti-p190 mAb, 8C10. D: GTP overlay assay. The same samples were separated on SDS–PAGE, transferred to a nitrocellulose membrane and probed with [α-^{32}P]GTP as described in Section 2. Longer exposure of the autoradiogram continued to show no [α-^{32}P]GTP binding in lane 9. These data are representative of two independent experiments.

4). By the lack of 8C10 binding to the control preparation (lane 5), contaminating proteins could be identified. Fig. 1C, lane 6 shows that the GTP binding mutant was also purified as a 50 kDa protein.

Fig. 1D depicts a GTP overlay assay in which protein preparations were subjected to SDS–PAGE and transferred to a nitrocellulose membrane in a buffer that allows for partial renaturation of the proteins [12]. Following incubation of the membrane with [α-^{32}P]GTP, it was observed that only the *wt* N-terminal domain binds GTP.

To determine the kinetics, dose-dependency and specificity of this binding, a GTP solution binding assay was employed, as described in Section 2. In these experiments, a partially purified fraction containing the α subunit of the heterotrimeric G protein, G_{i3}, was included as a positive control and the empty vector preparation was used as a negative control. As seen in Fig. 2A, the p190 N-terminal domain (residues 1–389) bound GTP with kinetics similar to the positive control and to a significantly greater extent than the negative control. Similar results were obtained with a GST fusion protein containing residues 29–240, which contains the core GTP binding sequences (data not shown, Fig. 1A and [19]). Fig. 2B shows that binding to the p190 N-terminal domain was dose-dependent and greater at all concentrations of nucleotide tested than the negative control. The GTP binding seen with the negative control likely represented a non-specific interaction, as the

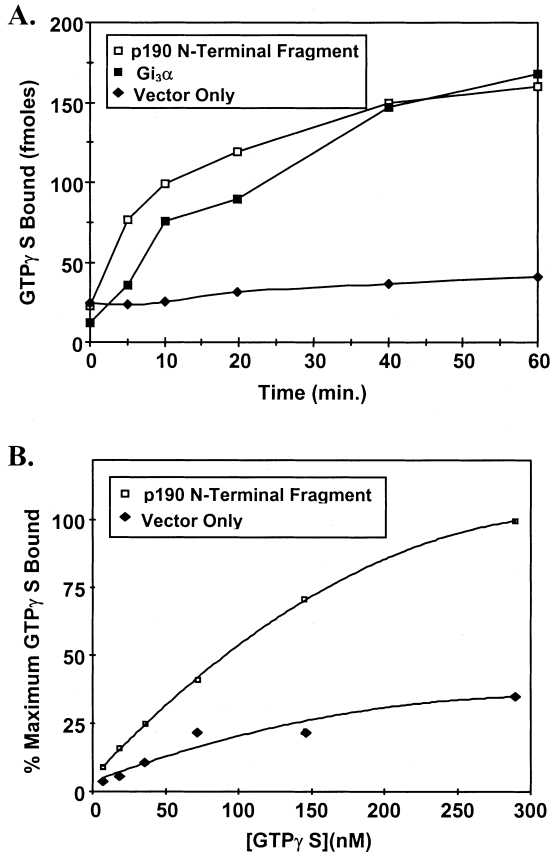

Fig. 2. Time-course and dose-dependency of GTP binding to the recombinant p190 N-terminal domain. A: Time-course of GTP binding. In a solution binding assay [γ-^{35}S]GTP was incubated with either 1 pmol p190 N-terminal domain (open squares), 1 pmol partially purified Gi3α subunit (closed squares) or a volume of vector-only contaminating proteins equal to that used for the p190 domain (closed diamonds) for the indicated times. The p190 domain bound GTP with a similar time-course and extent as Gi3α. At saturation, 0.15 mol GTP was bound per mol p190 or mol Gi3α, suggesting that ~15% of each protein preparation was conformationally active. Values represent the average of two data points. Similar results were obtained in two additional experiments, except that the amount of GTP bound at saturation varied from preparation to preparation. B: Dose-dependency of GTP binding. One pmol p190 N-terminal domain was incubated with indicated concentrations of unlabeled GTPγS for 60 min in a GTP solution binding buffer containing [γ-^{35}S]GTP, as described in Section 2. An equal volume of the vector-only contaminant proteins exhibited a background level of GTP binding. These data were reproduced in a separate experiment.

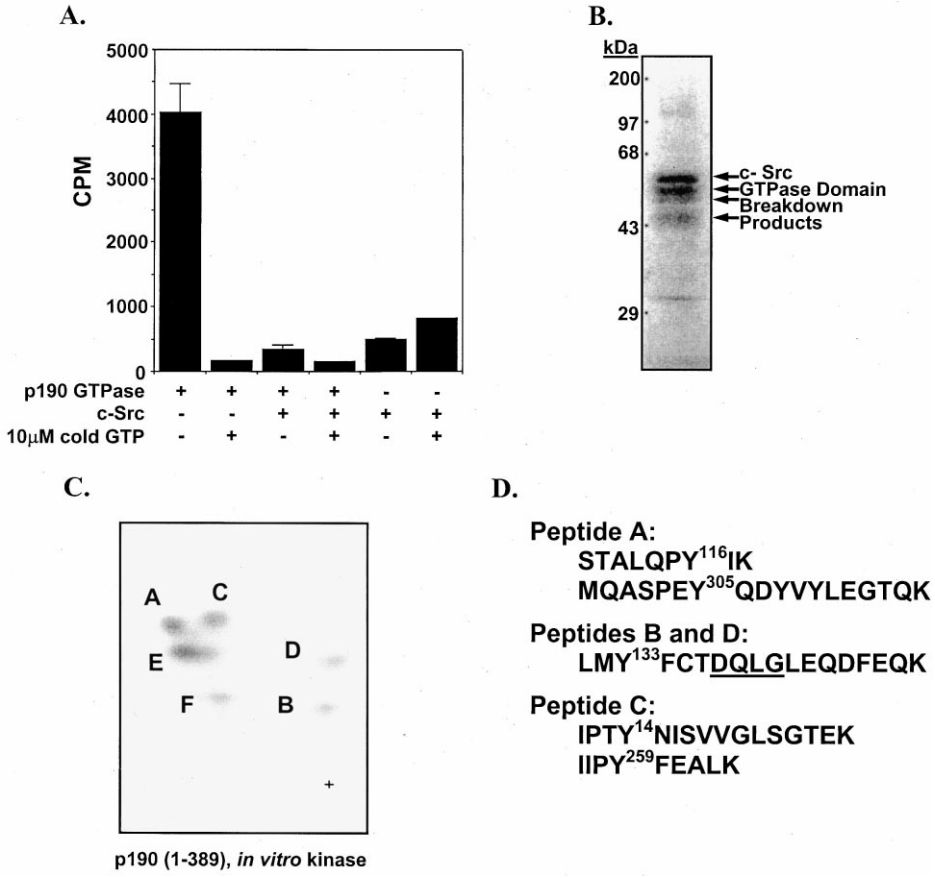

Fig. 3. Tyrosine phosphorylation of the p190 GTPase domain by c-Src inhibits GTP binding. A: GTP solution binding assay. Ni^{2+} resin-purified p190 N-terminal domain was incubated in kinase reaction buffer in the presence or absence of baculovirus expressed c-Src (UBI) and then assessed for the ability to bind [γ-^{35}S]GTP in the presence or absence of excess unlabeled GTP, as described in Section 2. This result was observed in two separate experiments, each performed in triplicate. B: Products of the kinase reaction. [γ-^{32}P]ATP (~1 μCi) was included in the in vitro kinase reaction and phosphorylated proteins were visualized by SDS–PAGE and autoradiography. C: Tryptic phosphopeptide map of the p190 N-terminal domain. The P190 N-terminal domain was phosphorylated in vitro by c-Src in the presence of [γ-^{32}P]ATP and subjected to tryptic phosphopeptide analysis as described in Section 2. ~2000 cpm were loaded on the TLC plate. D: Candidate phosphopeptides of the p190 N-terminal domain phosphorylated by c-Src. HPLC-purified tryptic phosphopeptides were subjected to sequential Edman degradation analysis and the cycle number at which maximum ^{32}P was released was matched to the tyrosine position within theoretical tryptic peptides of the p190 N-terminal domain, according to the method of Boyle et al. [15].

overlay assay ruled out the possibility that contaminant proteins had GTP binding activity. To assess the nucleotide specificity of binding, a competition study was carried out, using a panel of nucleotides as competitors for [γ-^{35}S]GTP binding. Table 1 shows that binding of [γ-^{35}S]GTP was effectively competed by cold, excess GTPγS, GTP and GDP but not by dGTP, GMP or other nucleotides or nucleosides. These results are similar to those obtained from nucleotide competition studies that examined the specificity of p21Ras GTPase activity [17]. Also consistent with previous studies in which full length p190 was examined [18], we were unable to detect by thin layer chromatography analysis a GTPase activity that was intrinsic to the isolated N-terminal domain (data not shown).

3.2. *Effect of Src phosphorylation on the p190 GTPase domain*

To examine the possibility that tyrosine phosphorylation could affect the ability of the p190 N-terminal domain to bind GTP, the recombinant His-tagged fusion protein was incubated in kinase buffer in the presence or absence of c-Src before subjecting it to a GTP solution binding assay as described in Section 2. Fig. 3A shows a greater than 90% decrease in the ability of the p190 N-terminal domain to bind [γ-^{35}S]GTP after phosphorylation by c-Src as compared to the N-terminal domain incubated in kinase buffer alone, while c-Src alone exhibited only background levels of GTP binding. In a separate kinase reaction, p190 was radiolabeled with [γ-^{32}P]ATP to assess phosphorylated products by autoradiography (Fig. 3B). Autophosphorylation of c-Src and transphosphorylation of the p190 N-terminus and several breakdown products were observed. Phosphoamino acid analysis [15] revealed that only tyrosine residues were phosphorylated (data not shown). Fig. 3C is a tryptic phosphopeptide map that demonstrates the complex nature of the phosphorylation pattern. Six phosphopeptides were routinely observed, and four (Fig. 3A–D) were successfully purified by HPLC and shown to co-migrate with tryptic phosphopeptides of in vivo ^{32}P-labeled p190 from v-Src transformed murine fibroblasts (data not shown). Fig. 3D lists the potential sites of phosphorylation by c-Src as determined by sequential Edman degradation of HPLC-isolated peptides and coincidence of the cycle number of peak ^{32}P release with the position of tyrosine residues in tryptic peptides derived from the N-terminal domain of p190. Peptides E and F were refractory to HPLC analysis.

4. Discussion

The data presented above demonstrate that the p190 N-terminal domain can be phosphorylated in vitro by c-Src, that the isolated, recombinant p190 protein binds GTP directly and specifically in a time- and dose-dependent manner and that phosphorylation by c-Src results in greater than 90% loss of GTP binding activity. While others have reported that full-length p190 binds GTP [18,19], this study is the first to describe an effect of tyrosine phosphorylation on GTP binding. Multiple sites are phosphorylated in the N-terminal domain by c-Src (Fig. 3C), but it is not known whether one or more of these sites is required for the effect or what the mechanism of inhibition may be. Y^{116} and Y^{133} are prime candidates for further investigation, since they reside within the GTP binding domain itself and Y^{133} is near the critical Mg^{2+} binding loop.

The stoichiometry of p190 N-terminal domain phosphorylation by c-Src was found to be $\sim$10%. Similarly, relatively low stoichiometries have previously been observed by us and others in studies with bacterially expressed α subunits of heterotrimeric G proteins [20]. Although we are uncertain as to the reason for these findings, we did experience some difficulties with the solubility of several p190 preparations, suggesting that only a portion of the protein was completely renatured following Ni^{2+} chromatography. However, the number of moles of phosphate incorporated into the p190 N-terminus was approximately equal to the number of moles of GTP bound to the unphosphorylated protein in these preparations, suggesting that the portion of p190 protein that assumed a native conformation was capable of either binding GTP or becoming phosphorylated.

Functional effects of tyrosine phosphorylation on other GTP-binding proteins have previously been described. For example, tyrosine phosphorylation mediated by the EGF receptor of a 22 kDa GTP binding protein (G25K) from brain was found to result in a small but significant increase in GTP binding when the protein was reconstituted into phospholipid vesicles [21]. In A431 cells, EGF treatment suppressed bradykinin-induced activation of the cAMP pathway via tyrosine phosphorylation of Gsα [22] and tyrosine phosphorylation of the Gq/G11 α subunit by v-Src was shown to lead to an increase in the ability of the α subunit to stimulate PLC-β in vitro [23]. In another study, phosphorylation of Gqα at Tyr 356 was found to be required for optimal inositol-1,4,5-trisphosphate (IP3) production following stimulation by multiple agonists of Gq/11-coupled receptors [24].

What role the GTP-binding domain of p190 plays in cell physiology is not yet clear. One potential role for the p190 GTP binding domain is to provide another site in the p190 molecule (in addition to the RhoGAP domain), by which tyrosine phosphorylation can affect actin cytoskeleton dynamics. As yet, no direct evidence for the involvement of the p190 GTP binding domain in this process has been forthcoming. However, the GTP binding domain of p190 has been shown to play a role in inducing cell morphology changes upon overexpression of full length p190 [19] and in reversing anchorage-independent growth of Ras-transformed cells [25]. Since both these processes involve changes in actin cytoskeleton structures, it is reasonable to speculate that the p190 GTP binding domain is involved in such events. In addition, recent studies in our laboratory suggest that the GTP binding domain is required, but not sufficient for p190-induced cell cycle arrest (Dukes et al., in preparation). Thus, the GTP binding domain of p190 could play a role in regulating several cellular processes, which in turn could be modulated by tyrosine phosphorylation.

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