Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 3343–3346, April 1992
Biochemistry

Ras GTPase-activating protein: A substrate and a potential binding protein of the protein-tyrosine kinase p56^{lck}

KURT E. AMREIN, NICHOLAS FLINT, BÄRBEL PANHOLZER, AND PAUL BURN

Department of Biology, Pharmaceutical Research New Technologies, F. Hoffmann–La Roche Ltd, CH-4002 Basel, Switzerland

Communicated by Gottfried Schatz, January 6, 1992

ABSTRACT **Ras GTPase-activating protein (GAP) is a cytoplasmic factor that regulates the GTPase activity of p21^{ras}. Phosphorylation of GAP on tyrosine has recently been reported by several groups and may be an important step in linking signaling pathways involving p21^{ras} and protein-tyrosine kinases. p56^{lck}, a src-like protein-tyrosine kinase, seems to play a crucial role in T-cell development and T-cell activation. However, the molecular mechanisms of T-cell signaling involving p56^{lck} and the substrates of p56^{lck} have not yet been identified. To test whether GAP is a substrate of p56^{lck}, *in vitro* kinase reactions were performed with purified, recombinant GAP and p56^{lck}. We found that GAP became specifically phosphorylated on tyrosine within one tryptic peptide. Furthermore, coimmunoprecipitation studies provided evidence that the tyrosine-phosphorylated form of GAP is bound to p56^{lck}. These results suggest that in T cells the function of GAP might be regulated through its phosphorylation on tyrosine and binding to the protein-tyrosine kinase p56^{lck}.**

GTPase-activating protein (GAP) is a cytoplasmic factor that greatly stimulates GTPase activity of normal p21^{ras} (1). To date, two different forms of GAP have been discovered; one with a mass of 120 kDa (p120-GAP) and the other with a mass of 95 kDa. The two forms of GAP are derived from two different RNA species that are generated by alternative splicing (2). GAP interacts with p21^{ras} protein at a site that is essential for Ras function (3). Recent reports have shown that GAP can be phosphorylated on tyrosine both by activated receptor kinases and by the constitutively activated protein-tyrosine kinases encoded by v-*fps* and v-*src* (4–6). In cells transformed by an activated protein-tyrosine kinase, GAP can associate with two tyrosine-phosphorylated proteins, p62 and p190 (6).

The *lck* gene encodes a 56-kDa protein-tyrosine kinase (p56^{lck}), which is expressed at high levels in T lymphocytes (7, 8). The structure of p56^{lck} is typical for src-like kinases. It shares a high sequence identity with p60^{src} within the kinase domain. A region of lesser, but still significant, homology is found between amino acids 65 and 185, which contain the src homology regions 2 and 3 (SH2 and SH3) (9, 10). p56^{lck} undergoes at least two posttranslational modifications: (*i*) myristoylation of the amino-terminal glycine residue, which directs p56^{lck} to the cytoplasmic face of the cell membrane (11, 12), and (*ii*) phosphorylation on tyrosine residues, which plays a role in the regulation of p56^{lck} protein-tyrosine kinase activity (13–15). In T cells, p56^{lck} is physically associated with the T-cell accessory molecules CD4 and CD8 (16–18). Therefore, p56^{lck} is believed to be important in processes such as the positive and negative selection of maturing T lymphocytes and the activation of functionally mature T lymphocytes.

Several cellular proteins have recently been described as substrates and binding proteins for activated receptor kinases such as the platelet-derived growth factor receptor or the epidermal growth factor receptor. Among these substrates are GAP, phospholipase Cγ, a subunit of phosphatidylinositol kinase 3 (p85), and the serine/threonine kinase Raf (19–24). Although there is no functional relationship between these proteins, all of these substrates seem to be important in intracellular signaling processes and three of them (GAP, phospholipase Cγ, p85) display two copies each of the SH2 domain (10). The SH2 domain is a sequence of ≈100 amino acids that was originally identified in protein-tyrosine kinases such as p60$^{c\text{-}src}$, p98^{fps}, and p145^{abl} (25). Proteins containing SH2 domains can interact with other proteins, most of which are phosphorylated on tyrosine residues. This has led to the view that SH2 domains are involved in recognition of specific phosphotyrosine residues (10).

The substrates of p56^{lck} have not yet been identified. Thus, in the present study we have investigated the possibility that p120-GAP can serve as a substrate for the nonreceptor tyrosine kinase p56^{lck} and that p120-GAP can interact with p56^{lck} *in vitro*. In contrast to other phosphorylation studies, purified soluble proteins were used in place of immunoprecipitated proteins. The possibility that p56^{lck} is either a regulator or an effector of GAP is discussed.

MATERIALS AND METHODS

Purified Recombinant Proteins. Recombinant human p56^{lck} was expressed in *Escherichia coli* using pDS expression vectors (Diagen, Düsseldorf, F.R.G.). p56^{lck} was affinity purified using a monoclonal anti-p56^{lck} antibody column. On a Coomassie blue-stained SDS/polyacrylamide gel, the protein was judged to be ≈10% pure. The activity of immunoprecipitated p56^{lck} isolated from *E. coli* or from T cells (Molt 4 cells) was comparable.

Recombinant human Ras-p120-GAP was a gift from Julie Scheffler and collaborators (Hoffmann–La Roche). It was purified from insect cells infected with a modified baculovirus strain encoding human p120-GAP. Analysis by SDS/PAGE revealed that the protein was >70% pure.

Antibodies and Antisera. Monoclonal mouse anti-p56^{lck} antibodies and polyclonal rabbit anti-p56^{lck} antiserum were raised against recombinant human p56^{lck} overexpressed in *E. coli*. Polyclonal rabbit anti-GAP antiserum was a gift from Frank McCormick (Cetus).

Kinase Assays. *In vitro* kinase reactions were performed in a total vol of 20 μl of kinase buffer (10 mM $MnCl_2$/0.1 μM ATP/50 mM Hepes, pH 6.8) for 10 min at 30°C. In standard kinase reaction mixtures were included 10 ng of partially purified recombinant p56^{lck} and, where appropriate, one or more of the following ingredients: tracer amounts of [γ-^{32}P]ATP (0.5–10 μCi per 20 μl; >5000 Ci/mmol; 1 Ci = 37 GBq; Amersham); 5 μg of acid-denaturated rabbit muscle enolase (Boehringer Mannheim) (26); 1 μg of p120-GAP. K_m values of p56^{lck} for enolase and p120-GAP were determined

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GAP, GTPase-activating protein; SH, src homology region.

at 1 μM ATP and at protein concentrations of 0.5–15 μg per 20 μl of assay mixture. Under these conditions, the assay was shown to be linear with time.

Tryptic Peptide Maps of p120-GAP. Tryptic peptide maps were prepared basically as described by Luo *et al.* (27). p120-GAP was labeled *in vitro* by $p56^{lck}$ in the presence of 10 μCi of [γ-^{32}P]ATP (>5000 Ci/mmol; Amersham). Labeled proteins resolved by SDS/PAGE were electrophoretically transferred to nitrocellulose and localized by fluorography, and the corresponding regions were cut out. Pieces of nitrocellulose containing labeled p120-GAP were soaked for 30 min in 100 mM acetic acid (37°C) containing 0.5% polyvinylpyrrolidone (PVP-360, Sigma) followed by digestion with trypsin. The eluted peptides (≈70% of total cpm) were oxidized with performic acid and then analyzed by two-dimensional phosphopeptide mapping on cellulose thin-layer plates (28). The first dimension was electrophoresis at pH 1.9 and the second dimension was ascending chromatography in phosphorylated chromatography buffer (*n*-butanol/pyridine/acetic acid/water, 75:50:15:60).

Phosphoamino Acid Analysis. Tryptic peptides isolated as described above were hydrolyzed in 5.7 M HCl for 1 h at 110°C. The hydrolysates were analyzed by two-dimensional thin-layer electrophoresis (29).

Coprecipitation Studies. Kinase reactions were performed as described above and stopped by adding 20 mM EDTA. The reactions were then split into three aliquots—A, B, and C. Aliquot B was diluted with 1 vol of 2× denaturation buffer (1% SDS/2 mM dithiothreitol/4 mM EDTA/100 mM Tris·HCl, pH 8.0) and then heated to 100°C for 3 min. Aliquots B and C were then diluted into 1 ml of ice-cold immunoprecipitation buffer (IP buffer; 1% Nonidet P-40/0.15 M NaCl/2 mM dithiothreitol/2 mM EDTA/0.01 M sodium phosphate, pH 7.2/1% Trasylol/2% gelatin). $p56^{lck}$ was precipitated specifically with 1 μl of polyclonal anti-$p56^{lck}$ antiserum and 20 μl of fixed *Staphylococcus aureus*. The immunoprecipitates were washed three times in IP buffer and once in TN buffer (0.15 M NaCl/50 mM Tris·HCl, pH 7.2). Finally, total protein (aliquot A) and immunoprecipitated protein (aliquots B and C) were analyzed by SDS/PAGE.

Anti-GAP Western Blot Analysis. Proteins resolved by SDS/PAGE were electrophoretically transferred to nitrocellulose membranes. Blocking and probing of the membranes were performed as described by Halenbeck *et al.* (2).

RESULTS

$p56^{lck}$ Phosphorylates p120-GAP on Tyrosine Within One Major Tryptic Peptide. To investigate whether p120-GAP is a direct substrate of the $p56^{lck}$ protein-tyrosine kinase, we studied p120-GAP phosphorylation by the protein-tyrosine kinase $p56^{lck}$ *in vitro*. Recombinant human p120-GAP and recombinant human $p56^{lck}$ were purified from baculovirus-infected insect cells and from a transformed *E. coli* strain, respectively.

In a first set of experiments, p120-GAP was incubated in kinase buffer in the presence or absence of soluble $p56^{lck}$ with [γ-^{32}P]ATP used as a phosphate donor (Fig. 1*a*). Phosphorylation of p120-GAP was observed when the protein-tyrosine kinase $p56^{lck}$ was present in the reaction mixture (Fig. 1*a*, lane 3), whereas no phosphorylation of p120-GAP was detectable in the absence of $p56^{lck}$ (lane 1) or in the presence of $p56^{lck}$ alone (lane 2). The weaker band of lower molecular weight observed in lanes 2 and 3 represents autophosphorylated $p56^{lck}$. Phosphoamino acid analysis of p120-GAP phosphorylated by $p56^{lck}$ demonstrated that phosphorylation occurred exclusively on tyrosine residues (Fig. 1*c*). Protein preparations of untransformed *E. coli* lysates in similar experiments did not display any detectable protein-tyrosine kinase activity (data not shown). We therefore conclude that the observed tyrosine phosphorylation of p120-GAP is due to $p56^{lck}$ activity. Tryptic peptide map analysis of *in vitro* phosphorylated p120-GAP displayed one major labeled phosphopeptide (Fig. 1*b*). Thus, although p120-GAP contains 38 tyrosine residues, it seems to be phosphorylated by the purified $p56^{lck}$ on a single tryptic peptide, indicating a high selectivity of $p56^{lck}$ for one particular tyrosine residue of p120-GAP.

In a second set of experiments, we performed *in vitro* kinase reaction experiments to determine the K_m of $p56^{lck}$ for the kinase substrate p120-GAP. $p56^{lck}$ exhibited a K_m of 2–3 μM for p120-GAP, which is similar to the K_m of $p56^{lck}$ for enolase (data not shown), one of the best *in vitro* substrates known. In kinase assays performed in the presence of both substrates (2 μg of enolase and 2 μg of p120-GAP per 20-μl reaction mixture), we found that enolase was phosphorylated ≈8 times better than p120-GAP, which is in agreement with the observed differences in the V_{max} values (data not shown). In addition, we noted that in the presence of p120-GAP phosphorylation of the exogenous substrate enolase was always slightly increased. For example, kinase reactions performed in a 5-fold excess of enolase over p120-GAP resulted in a significant and reproducible 2-fold stimulation of enolase phosphorylation (Fig. 2), as determined by Cerenkov counting of excised enolase bands. These results indicate that under specific conditions p120-GAP might be a regulator of $p56^{lck}$ kinase activity.

$p56^{lck}$ Forms a Complex with Phosphorylated p120-GAP. Recent reports have suggested that SH2 domains of proteins may be involved in protein–protein interactions by specifically recognizing and binding to phosphotyrosine-containing domains of other proteins (6, 19, 30, 31). Both $p56^{lck}$ and

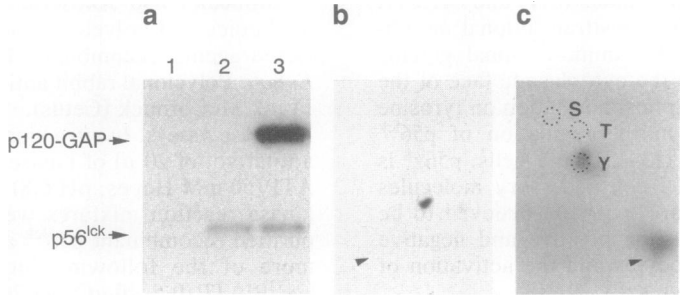

FIG. 1. $p56^{lck}$ phosphorylates p120-GAP on tyrosine within one major tryptic peptide. (*a*) *In vitro* kinase assays performed in the presence of p120-GAP alone (lane 1), $p56^{lck}$ alone (lane 2), and $p56^{lck}$ together with p120-GAP (lane 3). (*b*) Tryptic peptide map analysis of p120-GAP, phosphorylated by $p56^{lck}$ *in vitro*, was performed as described. In the horizontal direction, peptides were resolved by electrophoresis at pH 1.9 (positive electrode on the left) and in the vertical direction peptides were resolved by ascending chromatography. (*c*) Phosphoamino acid analysis of tryptic peptides of phosphorylated p120-GAP. The position of ninhydrin-stained phosphoamino acid standards is shown by dotted lines. Arrowheads mark positions of sample application.

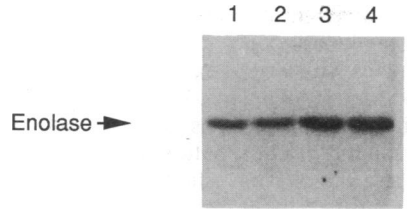

FIG. 2. p120-GAP stimulates $p56^{lck}$ kinase activity toward the exogenous substrate enolase. The phosphorylation of enolase (5 μg) by $p56^{lck}$ was measured in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of p120-GAP (1 μg) in the kinase reaction mixture (20 μl). $p56^{lck}$ or p120-GAP and $p56^{lck}$ were preincubated on ice for 10 min followed by addition of the substrate enolase for an additional 3 min at 30°C. The reaction mixture was then separated by SDS/PAGE and analyzed by fluorography. Note that the presence of p120-GAP doubled the phosphorylation of enolase (determined by Cerenkov counting of the excised bands).

p120-GAP display SH2 domains and both proteins can be phosphorylated on tyrosine residues. We therefore studied complex formation between the two SH2 domain-containing proteins by two different approaches.

In a first approach, p120-GAP (1 μg) and $p56^{lck}$ (10 ng) were incubated in kinase reaction buffer containing tracer amounts of [γ-^{32}P]ATP. After stopping the kinase reaction with EDTA, $p56^{lck}$ was immunoprecipitated from the p120-GAP/$p56^{lck}$ reaction mixture with anti-$p56^{lck}$ antiserum, separated by SDS/PAGE, and analyzed by fluorography (Fig. 3). Both phosphorylated p120-GAP and $p56^{lck}$ were present in the anti-$p56^{lck}$ immunoprecipitate (Fig. 3, lane 6). In several independent experiments, it was found that up to 80% of the phosphorylated p120-GAP present in the reaction mixture was coprecipitated with $p56^{lck}$ (Fig. 3).

Control experiments demonstrated that anti-$p56^{lck}$ antiserum precipitates p120-GAP because of its association with $p56^{lck}$ and not because of crossreactivity with p120-GAP or other unspecific interactions. First, anti-$p56^{lck}$ immunoprecipitates from kinase reaction mixtures boiled in 0.5% SDS prior to immunoprecipitation did not contain p120-GAP even though $p56^{lck}$ was precipitated efficiently (Fig. 3, lane 4). Second, if a preimmune serum was used then the precipitation of p120-GAP decreased in parallel with the precipitation of $p56^{lck}$ (lanes 7 and 8) to background level. Third, attempts to immunoprecipitate purified p120-GAP with anti-$p56^{lck}$ antiserum failed (data not shown).

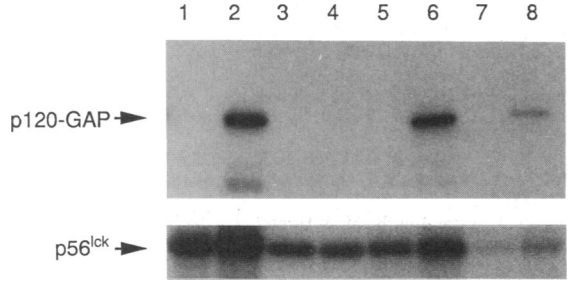

FIG. 3. Phosphorylated p120-GAP is coprecipitated with $p56^{lck}$ from *in vitro* kinase reaction mixtures. Two different kinase reaction mixtures containing $p56^{lck}$ alone (lanes 1, 3, 5, and 7) or $p56^{lck}$ and p120-GAP (lanes 2, 4, 6, and 8) were performed in the presence of [γ-^{32}P]ATP and then split into four aliquots. The first aliquot represents total protein (lanes 1 and 2). The other aliquots were diluted and immunoprecipitates were prepared using either anti-$p56^{lck}$ antiserum (lanes 3–6) or preimmune serum (lanes 7 and 8). The samples analyzed in lanes 3 and 4 were denaturated by boiling in 0.5% SDS before dilution and subjection to immunoprecipitation. Immunoprecipitated proteins and total proteins were separated by SDS/PAGE and were analyzed by fluorography. (*Upper*) Exposure was for 1 h. (*Lower*) Exposure was for 10 h.

In a second approach, the percentage of p120-GAP that coprecipitated with $p56^{lck}$ was determined. Kinase reactions were performed as described above except that unlabeled ATP was used in place of [γ-^{32}P]ATP. After immunoprecipitation of $p56^{lck}$ from the p120-GAP/$p56^{lck}$ reaction mixture and separation of the proteins by SDS/PAGE, the presence of p120-GAP in the immunocomplexes was analyzed by immunoblotting, with anti-GAP antiserum used as a probe (Fig. 4 *Left*). As judged from p120-GAP standards, which were analyzed in parallel (Fig. 4 *Right*), only $\approx$1% of the p120-GAP protein present in the kinase reaction mixture was specifically coprecipitated with $p56^{lck}$.

In summary, the experiments described in Fig. 4 suggest that a minor fraction of p120-GAP present in the kinase reaction mixture specifically interacts and coprecipitates with $p56^{lck}$. However, the experiments described in Fig. 3 showed that most of the phosphorylated p120-GAP coprecipitates with $p56^{lck}$. Thus, we conclude that $p56^{lck}$ is preferentially associated with the tyrosine phosphorylated form of p120-GAP.

DISCUSSION

Previous work indicated that $p56^{lck}$ is a key enzyme in the CD4- and CD8-mediated signal transduction cascade leading to T-cell activation (15–18, 32, 33). To elucidate the molecular mechanisms and the elements involved in this activation process, it is crucial to identify the substrates of $p56^{lck}$ as well as the potential regulators of $p56^{lck}$ kinase activity. In this study, an *in vitro* kinase assay system was used to investigate whether p120-GAP serves as a substrate for $p56^{lck}$. The results presented demonstrate that $p56^{lck}$ phosphorylates p120-GAP on a tyrosine residue within a single tryptic peptide and that the tyrosine phosphorylated form of p120-GAP can form a stable complex with $p56^{lck}$. Furthermore, we provide evidence that p120-GAP can stimulate the kinase activity of $p56^{lck}$ toward the exogenous substrate enolase.

Tyrosine phosphorylation of GAP has been previously observed. In intact cells, tyrosine phosphorylation of GAP can be induced by either epidermal growth factor or platelet-derived growth factor, or alternatively by the expression of

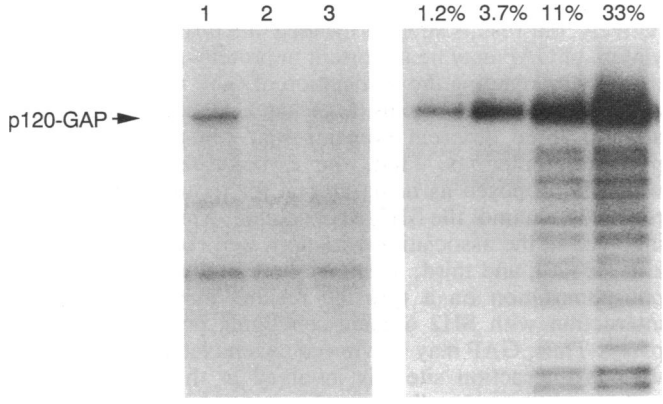

FIG. 4. Western blot analysis of the relative amount of total p120-GAP coprecipitated with $p56^{lck}$ from *in vitro* kinase reaction mixtures. From kinase reaction mixtures (*Left*) containing $p56^{lck}$ and an excess of p120-GAP, immunoprecipitations were performed with either anti-$p56^{lck}$ antiserum (lanes 1 and 2) or preimmune serum (lane 3). The sample in lane 2 was denaturated by boiling in 0.5% SDS before subjection to immunoprecipitation. In addition, p120-GAP standards (*Right*) were prepared containing 1.2–33% of the total amount of the p120-GAP protein present in a kinase reaction mixture. Both immunoprecipitated proteins and the p120-GAP standards were separated by SDS/PAGE and then analyzed by Western blotting, with a rabbit anti-GAP antiserum and ^{125}I-labeled protein A used as probes.

protein-tyrosine kinase oncogenes such as v-*src* (4–6, 19). In a recent report, phosphorylation of GAP on tyrosine residues was described in immunoprecipitation experiments involving $p60^{v\text{-}src}$ but not in immunoprecipitation experiments involving $p60^{c\text{-}src}$ (34). These results suggest that only the activated form of $p60^{src}$, $p60^{v\text{-}src}$, is able to phosphorylate GAP. In contrast, our results demonstrate that soluble, recombinant $p56^{lck}$ can phosphorylate p120-GAP, indicating that $p56^{lck}$, unlike $p60^{src}$, does not need to be activated by genetic alterations to be able to phosphorylate p120-GAP. However, it has to be considered that this finding may be explained by the fact that $p56^{lck}$ expressed in *E. coli* is mainly phosphorylated on Tyr-394 (data not shown), the autophosphorylation site, and therefore the kinase may be viewed to be in an activated form (13, 14). Our finding that p120-GAP is a direct substrate of $p56^{lck}$ is further substantiated by a recent study describing GAP tyrosine phosphorylation in fibroblasts overexpressing an activated form of $p56^{lck}$ ($p56^{lckF505}$) or in LSTRA cells expressing unusually high amounts of $p56^{lck}$ (35).

p120-GAP phosphorylated by $p56^{lck}$ yields one major phosphorylated tryptic peptide. In addition, CNBr analysis also resulted in identification of one major 18-kDa phosphopeptide (data not shown). The CNBr peptide of p120-GAP closest in size to 18 kDa corresponds to amino acids 444–569. Within this GAP fragment lies Tyr-460, which was recently identified as being phosphorylated by the activated epidermal growth factor receptor (5). These findings suggest that different protein tyrosine kinases might phosphorylate GAP specifically at the same position.

The only known biochemical function that has been attributed to GAP so far is its ability to enhance the relatively weak GTPase activity of normal $p21^{ras}$ (3, 36). Here we have provided evidence that p120-GAP might regulate the kinase activity of the protein-tyrosine kinase $p56^{lck}$. The observed activation of $p56^{lck}$ by p120-GAP is weak and could be due to the particular *in vitro* kinase conditions. However, it should be considered that *in vivo* other cellular components such as $p21^{ras}$, p62, and p190, which can interact with GAP in cellular systems, might be able to potentiate the observed stimulation.

Tyrosine phosphorylation of GAP might change its effect on the GTPase activity of $p21^{ras}$. To date, however, there are no data available that would support such a theory. Alternatively, our results support the idea that tyrosine phosphorylation of GAP may be important in protein–protein interactions by modulating the association of GAP with other SH2 domain-containing proteins (6). Thus, GAP seems to display at least three different domains that can direct protein–protein interactions. First, the carboxyl-terminal region, which is proposed as being crucial for its interaction with $p21^{ras}$ (3); second, the SH2/SH3 domain, which seems to be involved in the association with activated receptor tyrosine kinases (20); and third, as shown here, a domain that upon phosphorylation on a tyrosine residue may stabilize the interaction with SH2 domain-containing proteins such as $p56^{lck}$. Thus, GAP may be viewed as a molecule that, by its various interaction sites, is involved in the formation of multicomponent signaling complexes.

Available evidence suggests that $p21^{ras}$ has an essential function in mitogenic signaling downstream of growth factor receptors and oncoproteins with tyrosine kinase activity (37, 38). Our findings that $p56^{lck}$ phosphorylates and binds to tyrosine-phosphorylated p120-GAP *in vitro* indicates that $p56^{lck}$ may regulate $p21^{ras}$ function *in vivo* indirectly through phosphorylation of and binding to GAP. Alternative to the idea that $p56^{lck}$ is a regulator of GAP function, $p56^{lck}$ could be an effector of GAP since we have provided some evidence that p120-GAP can stimulate $p56^{lck}$ protein-tyrosine kinase activity. In either case, in T cells GAP might be viewed as a link between signal transduction pathways involving the protein-tyrosine kinase $p56^{lck}$ and the G protein $p21^{ras}$.

Further studies will need to examine the exact relationship between $p56^{lck}$ and GAP *in vivo*. In addition, the role of other SH2 domain-containing proteins, such as phospholipase Cγ and the p85 subunit of phosphatidylinositol 3-kinase as substrates or regulators of $p56^{lck}$ should be addressed.

We are grateful to Alexander Wood, Julie Scheffler, Frank McCormick, and collaborators for providing us with purified p120-GAP and anti-GAP antiserum. We thank Michael Steinmetz, Martin Gassmann, and Alexander Wood for critical reading and Patricia Sayer for preparation of the manuscript.

1. Trahey, M. & McCormick, F. (1987) *Science* **238**, 542–545.
2. Halenbeck, R., Crosier, W. J., Clark, R., McCormick, F. & Koths, K. (1990) *J. Biol. Chem.* **265**, 21922–21928.
3. Adari, K., Lowy, D. R., Willumsen, B. M., Der, C. J. & McCormick, F. (1988) *Science* **240**, 518–521.
4. Molloy, C. J., Bottaro, D. P., Fleming, T. P., Marshall, M. S., Gibbs, J. B. & Aaronson, S. A. (1989) *Nature (London)* **342**, 711–714.
5. Xianquan, L. & Pawson, T. (1991) *Mol. Cell. Biol.* **11**, 2511–2516.
6. Ellis, C., Moran, M., McCormick, F. & Pawson, T. (1990) *Nature (London)* **343**, 377–381.
7. Voronova, A. F. & Sefton, B. M. (1986) *Nature (London)* **319**, 682–685.
8. Marth, J. D., Peet, R., Krebs, E. G. & Perlmutter, R. M. (1985) *Cell* **43**, 393–404.
9. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930.
10. Koch, A. C., Anderson, D., Moran, M. F., Ellis, C. & Pawson, T. (1991) *Science* **252**, 668–674.
11. Voronova, A. F., Buss, J. E., Patschinsky, T., Hunter, T. & Sefton, B. M. (1985) *Mol. Cell. Biol.* **4**, 2705–2713.
12. Abraham, N. & Veillette, A. (1990) *Mol. Cell. Biol.* **10**, 5197–5206.
13. Amrein, K. E. & Sefton, B. M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4247–4251.
14. Marth, J. D., Cooper, J. A., King, C. S., Ziegler, S. F., Tinker, D. A., Overell, R. W., Krebs, E. G. & Perlmutter, R. M. (1988) *Mol. Cell. Biol.* **8**, 540–550.
15. Veillette, A. & Fournel, M. (1990) *Oncogene* **5**, 1455–1462.
16. Rudd, C. E., Trevillyan, J. M., Dasgupta, J. D., Wong, L. L. & Schlossman, S. F. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5190–5194.
17. Veillette, A., Bookman, M. A., Horak, E. M. & Bolen, J. B. (1988) *Cell* **44**, 301–308.
18. Shaw, A. S., Amrein, K. E., Hammond, C., Stern, D. F., Sefton, B. M. & Rose, J. K. (1989) *Cell* **59**, 627–636.
19. Kaplan, D. R., Morrison, D. K., Wong, G., McCormick, F. & Williams, L. T. (1990) *Cell* **61**, 125–133.
20. Margolis, B., Rhee, S. G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A. & Schlessinger, J. (1989) *Cell* **57**, 1101–1107.
21. Meisenhelder, J., Suh, P.-G., Rhee, S. G. & Hunter, T. (1989) *Cell* **57**, 1109–1122.
22. Morrison, D. K., Kaplan, D. R., Escobedo, J. A., Rapp, U. R., Roberts, T. M. & Williams, L. T. (1989) *Cell* **58**, 649–657.
23. Escobedo, J. A., Navankasattusas, S., Milfay, D., Ffried, V. A. & Williams, L. R. (1991) *Cell* **65**, 75–82.
24. Choudhury, G. G., Sylvia, V. L., Pfeifer, A., Wang, L. M., Smith, E. A. & Sakaguchi, A. Y. (1990) *Biochem. Biophys. Res. Commun.* **172**, 154–159.
25. Pawson, T. (1988) *Oncogene* **3**, 491–495.
26. Cooper, J. A., Esch, F., Taylor, S. S. & Hunter, T. (1984) *J. Biol. Chem.* **259**, 7835–7841.
27. Luo, K. X., Hurley, T. R. & Sefton, B. M. (1990) *Oncogene* **5**, 921–923.
28. Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 1311–1315.
29. Cooper, J. A., Sefton, B. M. & Hunter, T. (1983) *Methods Enzymol.* **99**, 387–402.
30. Mayer, B. J. & Hanafusa, H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2638–2642.
31. Matsuda, M., Mayer, B. J., Fukui, Y. & Hanafusa, H. (1990) *Science* **248**, 1537–1539.
32. Veillette, A., Sleckman, B. P., Ratnofsky, S., Bolen, J. B. & Burakoff, S. J. (1990) *Eur. J. Immunol.* **20**, 1397–1400.
33. Luo, K. X. & Sefton, B. M. (1990) *Mol. Cell. Biol.* **10**, 5305–5313.
34. Brott, B. K., Decker, S., Shafer, J., Gibbs, J. B. & Jove, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 755–759.
35. Ellis, C., Xingquan, L., Anderson, D., Abraham, N., Veillette, A. & Pawson, T. (1991) *Oncogene* **6**, 895–901.
36. McCormick, F. (1989) *Cell* **56**, 5–8.
37. Mulcahy, L. S., Smith, M. R. & Stacey, D. W. (1985) *Nature (London)* **313**, 241–243.
38. Smith, M. R., DeGudicibus, S. J. & Stacey, D. W. (1986) *Nature (London)* **320**, 540–543.