Phosphorylation of rap1GAP in Vivo and by cAMP-dependent Kinase and the Cell Cycle p34\(^{cdk2}\) Kinase in Vitro*

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Krev-1/rap1 is a member of an increasingly large family of ras-related proteins all sharing the ability to bind and hydrolyze GTP and to undergo a carboxyl-terminal isoprenylation modification. These proteins localize to a variety of subcellular membrane compartments and appear to function in a wide array of regulatory processes (reviewed in Refs. 1–3). By analogy to p21\(^{ras}\), the active state of p21\(^{rap}\) is probably dependent upon its binding GTP, while hydrolysis to GDP terminates this activity. Although p21\(^{rap}\) possesses a weak intrinsic GTPase activity, there exist regulatory proteins, termed GAPS for GTPase activating proteins, that specifically stimulate its GTP hydrolytic rate (4, 5). A cDNA coding for a GAP specific for the rap1 protein was recently cloned (6). This GAP exhibits no homology to the previously cloned rasGAP but is distinct from the product of SK-MEL-3 cells with dibutyryl CAMP promoted phosphorylation of rap1GAP in vivo. Based on the results of comparative phosphopeptide mapping the sites of phosphorylation in vivo and in vitro are identical.

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Materials—\(\gamma\)-\[^{32}\text{P}\]ATP (3000 Ci/mmol) was from Du Pont-New England Nuclear; \[^{32}\text{P}\]H\(_2\)PO\(_4\) (285 Ci/mg) was from ICN, Costa Mesa, CA; \[^{35}\text{S}\]methionine (1134 Ci/mmg) was from Amersham Corp.; dibutyryl cAMP, the catalytic subunit of cAMP-dependent kinase, phosphoamino acid standards, and cyanoacylamide gels were purchased from Sigma; trypsin, chymotrypsin, and endoproteinase Glu-C were purchased from Calbiochem (La Jolla, CA); and \[^{3}H\]GTP was from Du Pont.

MATERIALS AND METHODS

The abbreviations used are: GAP, GTPase activating protein; cAMP-PK, cAMP-dependent protein kinase; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; CAPS, 3-(cyclohexylamino)propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)methyl]glycine.

The complete lack of structural identity between GAPS specific for the different GTP-binding proteins suggests they function in disparate signaling systems in the cell. The identification of proteins, enzymes, or second messengers that associate with or modify GAPS will help to assemble the signaling networks in which the various GTP-binding proteins operate. For example, the finding that rasGAP is phosphorylated by growth factor receptors (8, 9) supports the proposal that p21\(^{ras}\) itself is coupled to growth factor receptor signaling (10). It is conceivable that rap1GAP is also coupled to cell signaling events which have already been extensively characterized. As a first step toward understanding the signaling systems affecting rap1GAP we have investigated the nature of a previously reported post-translational modification of the protein (6). This modification, manifested as a multiple banding pattern on SDS-polyacrylamide gels, was detected with both the recombinantly expressed protein and lysates from mammalian tissues and cell lines (6).

Since phosphorylation is known to affect the mobility of proteins on SDS gels, and the rap1GAP amino acid sequence contains strong consensus sites for phosphorylation, we sought to determine whether rap1GAP was a phosphoprotein. Here we report that rap1GAP is substantially phosphorylated on serine residues in vivo and that this phosphorylation is enhanced by reagents that activate cAMP-dependent kinase. We also demonstrate that both cAMP-dependent kinase and the cell cycle p34\(^{cdk2}\) kinase phosphorylate rap1GAP quantitatively in vitro at sites proximal to each other in the poly-peptide chain.

EXPERIMENTAL PROCEDURES

Materials—\(\gamma\)-\[^{32}\text{P}\]ATP (3000 Ci/mmol) was from Du Pont-New England Nuclear; \[^{32}\text{P}\]H\(_2\)PO\(_4\) (285 Ci/mg) was from ICN, Costa Mesa, CA; \[^{35}\text{S}\]methionine (1134 Ci/mmg) was from Amersham Corp.; dibutyryl cAMP, the catalytic subunit of cAMP-dependent kinase, phosphoamino acid standards, and cyanoacylamide gels were purchased from Sigma; trypsin, chymotrypsin, and endoproteinase Glu-C were purchased from Calbiochem (La Jolla, CA); and \[^{3}H\]GTP was from Du Pont.

Expression and Purification of Recombinant rap1GAP—Insect Sf9 cells were infected with recombinant baculovirus carrying the HuB10-A rap1GAP cDNA as previously described (6). Approximately 100 ml of cell suspension was centrifuged and the pellets lysed by freeze-thawing in 10 ml of 20 mM Tris, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, and pepstatin at 1 \(\mu\)g/ml. Following centrifugation at 100,000 \(\times g\) for 1 h the supernatant was adjusted to pH 6.5 and chromatographed on S-Sepharose (Pharmacia LKB Biotechnology Inc.) equilibrated in 50 mM sodium phosphate, pH 6.5, containing 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, and pepstatin at 1 \(\mu\)g/ml. The column was eluted with a 100-ml gradient of 0–300 mM NaCl, at a flow rate of 20 ml/h. Peak fractions containing rap1GAP activity were chromatographed on a 500 ml S-300 Sephacryl
column (Pharmacia LKB) equilibrated in 25 mM Tris, pH 8.0, containing 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, and pepstatin at 1 μg/ml. Peak fractions were pooled and concentrated to approximately 0.5 mg/ml total protein. These preparations were judged to be greater than 95% pure were pooled and concentrated to approximately 0.5 mg/ml total column (Pharmacia LKB) equilibrated in 25 mM Tris, pH 8.0, containing 1 mM dithiothreitol, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, and pepstatin at 1 μg/ml. For labeling SK-MEL-3 cells with 32P, cells were labeled at confluency in 60-mm dishes essentially as described above for Sf9 cells. For treatment with dibutyryl cAMP, cells were incubated for 2 h with 2 mM dibutyryl cAMP beginning at the time of addition of 32P.

Phosphorylation of rap1GAP in Vitro—Kinase reactions were carried out in a 100-μl final volume containing 25 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 50 mM [γ-32P]ATP (10,000-20,000 cpm/pmole), and 5 μg of purified rap1GAP. Reactions were started by the addition of either 10 ng of cAMP-PK or 2 ng of purified p34

Phosphoamino Acid Analysis—Phosphorylated rap1GAP was immunoprecipitated from lysates of 32P-labeled Sf9 cells overexpressing rap1GAP. Immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis and then electroblotted to a PVDF filter membrane. The radiolabeled rap1GAP was excised from the blot and then hydrolyzed by vapor phase contact with 6 N HCl at 110 °C for 2 h essentially as described by Hildebrandt and Fried (11). The amino acids were eluted from the membrane with distilled water and standard phosphoamino acids (5 nmol each) were added to the sample prior to chromatography. Phosphoamino acids were resolved by thin layer chromatography using the ammonia/ethanol solvent system described by Munoz and Marshall (12). Standard amino acids were visualized by staining with ninhydrin and 32P-labeled residues were detected by autoradiography.

Electrophoretic and Immunologic Procedures—For the separation and Western blotting of intact rap1GAP, SDS-polyacrylamide gel electrophoresis was performed in 8% gels essentially as described by Laemmli (13). Digestions of rap1GAP were resolved on 16% SDS-polyacrylamide gels using the Tricine gel system described by Chagger and Von Jagow (14). Western blotting for detection of rap1GAP with antisera was performed using nitrocellulose filters and 20 mM Tris, 192 mM glycine, 20% methanol. Electrophoresis for amino acid sequencing of resolved peptides was performed using PVDF filters and 16% CAPS buffer, pH 11.0, containing 10% methanol as described by Matsudaira (15). Antibodies specific to rap1GAP were raised in rabbits against the purified recombinant protein as previously described (6). The sera was affinity-purified against immobilized purified rap1GAP and diluted to a stock concentration of 0.25 mg/ml. For Western blotting, the purified antibody was used at a dilution of 1/10,000. Antibody immunoprecipitations were added to 500 μl of cell lysate and incubated at 4 °C overnight. Immunocomplexes were recovered using protein A-Sepharose. Western blots were developed using goat anti-rabbit IgG coupled to horseradish peroxidase (Bio-Rad, Richmond, CA) and visualized using the ECL system (Amer sham, UK).

Proteolytic and Chemical Digestion of rap1GAP—For the digestion of rap1GAP by CNBr, 25 μg of the purified rap1GAP was phosphorylated in vitro and then precipitated on ice with trichloroacetic acid (20% final volume). The protein was redissolved in 70% formic acid containing 30 mg/ml CNBr and digested overnight at room temperature. The sample was evaporated, redissolved in SDS-gel sample buffer, and applied to the gel. For enzymatic digestions, the procedure described by Cleveland (16) was followed. Five μg of the phosphorylated rap1GAP was boiled for 2 min in the presence of 100 mM Tris, pH 6.8, 0.5% SDS, 10 mM dithiothreitol, 10% glycerol, and the indicated proteases were added to a final concentration of 50 μg/ml. Digestions were carried overnight at room temperature, and the samples were boiled in SDS-gel sample buffer prior to electrophoresis.

Other Procedures—Protein concentration of rap1GAP was determined by amino acid analysis on a Beckman System 6300 high performance analyzer. Amino acid sequencing was performed on an Applied Biosystems model 470A gas phase automatic sequenator.

RESULTS
To determine whether rap1GAP was phosphorylated in vivo we expressed rap1GAP cDNA in insect Sf9 cells using the baculovirus expression system. The cells were metabolically labeled with either 32P or [35S]methionine, and lysates were subjected to SDS-polyacrylamide gel electrophoresis. An autoradiogram shows that a lysate from the Sf9 cells infected with the rap1GAP recombinant virus contained an approximate 85-kDa phosphoprotein not detected in the lysates from cells infected with a control virus (Fig. 1A). The lysate from the [35S]methionine-labeled cells contained a doublet with a molecular mass of 80-85 kDa not seen in the control lysate (Fig. 1A). On lower exposures only a single band, corresponding to the upper band detected with [35S]methionine labeling, could be detected in the phosphate-labeled sample (not shown). We next carried out phosphoamino acid analysis of the 32P-labeled rap1GAP. Following immunoprecipitation of rap1GAP from the Sf9 cell lysate, the protein was electroblotted from an SDS-gel and the immobilized protein was hydrolyzed with HCl. A chromatogram of the hydrolysate revealed that most radioactivity comigrated with the phosphoamino acids standard, while a lower level of phosphothreonine was also detected (Fig. 1B). A trace amount of radioactivity was also detected near the top of the chromatogram but migrated slightly faster than the phosphotyrosine standard.

The deduced amino acid sequence of rap1GAP contains some of the most commonly identified recognition sequences for phosphorylation by cAMP-dependent protein kinase (reviewed in Refs. 17 and 18). To test whether rap1GAP was a substrate for this kinase we incubated purified recombinant rap1GAP with the catalytic subunit of cAMP-dependent kinase (cAMP-PK) in the presence of [γ-32P]ATP. rap1GAP overexpressed in insect cells is phosphorylated on serine residues. A, insect Sf9 cells infected with baculovirus carrying a deleted polyhedrin promoter (lanes 1 and 3) or the rap1GAP cDNA (lanes 2 and 4) were metabolically labeled with either ortho-32P (lanes 1 and 2) or [35S]methionine (lanes 3 and 4). Cell lysates were subjected to SDS-gel electrophoresis and autoradiography. Arrow indicates the position of rap1GAP and values at left are molecular weights (× 10^-3) of standard proteins. B, phosphoamino acid analysis of rap1GAP immunoprecipitated from the insect cell lysate. Details are described under “Experimental Procedures.”

FIG. 1. rap1GAP overexpressed in insect cells is phosphorylated on serine residues.

- A, insect Sf9 cells infected with baculovirus carrying a deleted polyhedrin promoter (lanes 1 and 3) or the rap1GAP cDNA (lanes 2 and 4) were metabolically labeled with either ortho-32P (lanes 1 and 2) or [35S]methionine (lanes 3 and 4). Cell lysates were subjected to SDS-gel electrophoresis and autoradiography. Arrow indicates the position of rap1GAP and values at left are molecular weights (× 10^-3) of standard proteins. B, phosphoamino acid analysis of rap1GAP immunoprecipitated from the insect cell lysate. Details are described under “Experimental Procedures.”
was rapidly phosphorylated and maximally incorporated approximately 3 mol of phosphate/mole of protein (Fig. 2). The incorporation of phosphate was also reflected in a shift in mobility of rap1GAP on SDS-polyacrylamide gels (Fig. 2, inset). To localize the site of phosphorylation by cAMP-PK the radiolabeled protein was digested with CNBr and the fragments resolved by gel electrophoresis. A single 13.5-kDa digestion product contained the majority of the radioactivity (Fig. 3A). Amino acid sequencing of this peptide yielded the sequence shown underlined in Fig. 3B. This sequence immediately follows methionine residue 445 in the rap1GAP sequence and the next methionine COOH-terminal to this is found at position 545. The predicted molecular mass of this peptide is comparable to that determined for the CNBr fragment on SDS-polyacrylamide gels. Consistent with the phosphorylation of this region by cAMP-PK is the identification of several consensus recognition motifs for this kinase. In particular, the most commonly identified motif, R-R-K-X/S/T, is represented twice and the R-X-S/T motif once within the phosphorylated rap1GAP fragment.

The rap1GAP sequence also contains consensus sites for the cell cycle p34cdc2 kinase (17, 18). When incubated with purified p34cdc2, rap1GAP again rapidly incorporated phosphate. The maximal incorporation reached a plateau at approximately 2 mol of phosphate/mole of rap1GAP (Fig. 4). Following a 90-min incubation with p34cdc2 the subsequent addition of cAMP-PK resulted in further rapid phosphorylation by cAMP-PK.

The rap1GAP sequence was divided and incubated further with either CAMP-PK or a second addition of p34cdc2 (Fig. 4). This demonstrates that the sites of phosphorylation for these two kinases are distinct. To compare the sites of phosphorylation we phosphorylated rap1GAP using p34cdc2 or cAMP-PK and digested the radiolabeled proteins with CNBr. The protein phosphorylated by p34cdc2 generated a 13.5-kDa radiolabeled fragment identical in mobility to that produced from the rap1GAP phosphorylated by cAMP-PK (Fig. 5). Thus, the sites of phosphorylation by p34cdc2 are also within the carboxyl-terminal region located between methionine residues 445 and 545. Within this region there are several S/T residues immediately followed by prolines; a requirement for tryptic digestion of the CAMP-PK-labeled rap1GAP but not with the protein labeled by p34cdc2 (Fig. 5). We further compared the sites of phosphorylation by carrying out limited enzymatic digestions of rap1GAP phosphorylated by the two kinases. This treatment resulted in the generation of a set of overlapping radiolabeled peptides that were resolved by SDS-polyacrylamide gel electrophoresis (Fig. 5). Nearly identical peptide maps were generated from the rap1GAP phosphorylated by p34cdc2 or cAMP-PK. Again, this suggests that although the sites of phosphorylation by these two kinases are distinct they are likely very near each other in the polypeptide chain.
rap1GAP phosphorylated in vivo (see below and Fig. 7B).

To confirm that the major sites of phosphorylation were localized to the carboxyl-terminal region of rap1GAP, a deletion mutant lacking the carboxyl-terminal one-third of the protein was generated. This mutant, designated RG12, was produced in the Sf9 cell, purified to near homogeneity, and protein was generated. This mutant, designated RG12, was not phosphorylated

Fig. 6. A rap1GAP carboxyl-terminal truncation mutant is not phosphorylated in vitro. A rap1GAP mutant lacking residues 443–663 and designated RG12 was produced and purified as described under “Experimental Procedures.” A, linear representation showing the relationship of RG12 to full-length rap1GAP. The phosphorylated region derived from CNBr digestion at methionine residues 445 and 545 is also indicated. B, comparative phosphorylation in vitro of RG12 (circles) and full-length rap1GAP (squares) by either cAMP-PK (open symbols) or p34 kinase (closed symbols).

Of full-length rap1GAP (data not shown). Nevertheless, the phospho- proteins, radiolabeled either in vivo or in vitro, generated the same set of polypeptides on digestion with chymotrypsin (Fig. 7B). These results demonstrate that rap1GAP is phosphorylated in vivo at sites that are proximal or identical to those phosphorylated in vitro by cAMP-PK and p34 kinase. One exception is the 4-kDa phosphopeptide obtained from digestion of rap1GAP phosphorylated by cAMP-PK in vitro but not in vivo. This is the case even for cells stimulated with dibutyryl cAMP. As noted above, this suggests that the site of phosphorylation contained in this 4-kDa peptide may not be relevant to phosphorylation as it occurs in the cell. In this experiment we also digested rap1GAP that was phosphorylated by CAMP-PK and p34 kinase. No new digestion products were noted in comparison to those produced from rap1GAP phosphorylated by either kinase alone (Fig. 7B).

DISCUSSION

When expressed in recombinant systems rap1GAP undergoes a post-translational modification that affects its mobility on SDS-polyacrylamide gels (6). Multiple forms of rap1GAP, differing in apparent molecular mass, were also consistently identified in lysates from various mammalian tissues and cell lines (6). Here we have shown that these multiple forms of rap1GAP are at least in part due to phosphorylation of the protein on serine residues. Although the exact stoichiometry of phosphorylation in vivo was not determined, the relative intensity of the multiple forms of rap1GAP detected in Western blots suggests that a substantial percentage of the protein exists in the phosphorylated state in the cell. The multiple banding pattern is apparently indicative of phosphorylation, because the relative intensities of the bands were altered following treatment of the cells with an activator of cAMP-PK. Moreover, this shift in mobility of rap1GAP on SDS-gels

3 B. Rubinfeld, P. Polakis, and F. McCormick, unpublished data.
was reproduced in vitro by phosphorylation of the purified protein with cAMP-PK.

From the in vitro phosphorylation data it is clear that rap1GAP incorporates several stoichiometric equivalents of phosphate. The molar ratio of phosphate to rap1GAP ranged from 2 to 3 using cAMP-PK and was as high as 5 in some experiments where both cAMP-PK and p34<sup>cdc2</sup> were employed. The maximum level to which rap1GAP could be phosphorylated varied to some degree across preparations and was likely dependent upon the relative level of phosphorylation pre-existing in the purified rap1GAP. During the purification of the recombinant protein used in these studies we biased the yields for the lowest molecular mass forms of rap1GAP in an attempt to enrich for the nonphosphorylated forms. However, slower migrating forms, presumably phosphorylated, were still evident in the final preparations. Moreover, the shift in mobility observed on SDS-gels following phosphorylation of rap1GAP in vitro did not occur until an approximate molar ratio of 2 was achieved. Therefore, even the fastest migrating forms of rap1GAP may nevertheless be substantially phosphorylated. In any event, it is clear that rap1GAP undergoes hyperphosphorylation in vitro and based on comparative phosphopeptide mapping this is also likely the case for phosphorylation in vivo.

The carboxyl-terminal region of rap1GAP contains the sites of phosphorylation by cAMP-PK and p34<sup>cdc2</sup>. These phosphorylation sites were localized to an approximate 13.5-kDa fragment generated by digestion with CNBr. This segment of the rap1GAP primary structure, bordered by methionine residues 445 and 545, contains 24 threonine and serine residues. From our data it cannot be determined which of these residues are phosphorylated by cAMP-PK. However, 3 of these serines are situated with basic residues at the -2 and/or -3 position with respect to the putative phosphate acceptor: a requirement for recognition by cAMP-PK (17, 18). Moreover, two of these, Ser<sup>480</sup> and Ser<sup>489</sup>, comply with the most typical motif for recognition by cAMP-PK (17, 18). Phosphorylation in vitro by p34<sup>cdc2</sup> also occurs within this same serine/threonine-rich segment of rap1GAP. A primary recognition sequence for p34<sup>cdc2</sup> has not been well defined, but in all cases there is a requirement for a proline residue immediately carboxyl-terminal to the Ser/Thr phosphate acceptor (17, 18). Most of the reported sites for p34<sup>cdc2</sup>, such as those identified in T-antigen (19), p60<sup>src</sup> (20), the rab 1A and 4 proteins (21), nucleolin (22), and nuclear lamin proteins (23) also contain basic residues 1 residue carboxyl-terminal to the essential proline. The phosphorylated CNBr fragment of rap1GAP contains 4 Ser/Thr-Pro pairs, and one of these, Ser<sup>489</sup>, is followed by a basic residue in this context. In fact, the rap1GAP S<sup>489</sup>PTPR motif is identical to that conserved in the amino-terminal tail of all lamin proteins (20) and a 17-mer peptide containing this SPTP motif inhibits phosphorylation of lam and H1 histone by p34<sup>cdc2</sup> (23).

The proposed sites of phosphorylation are also consistent with the peptide mapping results. When digested with three different proteases, nearly identical sets of phosphopeptides were generated from rap1GAP phosphorylated by either p34<sup>cdc2</sup> or cAMP-PK. The observation that most of the peptides produced contained both a p34<sup>cdc2</sup> site and a cAMP-PK site suggests that extensive primary structure does not lie between the phospho-acceptor sites for the two kinases. In addition, the inability to phosphorylate the carboxyl-terminal truncation mutant, RG12, indicates that the sites of phosphorylation are clustered within the same general region of rap1GAP primary structure.

The significance of phosphorylation of rap1GAP by either cAMP-PK or p34<sup>cdc2</sup> is not yet understood. A significant enhancement of rap1GAP activity toward p21<sup>r<sup>61</sup></sup> was not observed when rap1GAP was phosphorylated in vitro. However, it is more difficult to appreciate how phosphorylation might affect rap1GAP activity in vivo. Phosphorylation of rap1GAP in vivo may influence its interaction with other proteins or second messengers, which in turn could have a profound affect on its activity toward p21<sup>r<sup>61</sup></sup>. Alternatively, the association of rap1GAP with a particular subcellular component may be influenced by its state of phosphorylation. This has been proposed for a number of regulatory proteins including p21<sup>r<sup>61</sup></sup> itself (24). rap1GAP is detected both in membrane and cytosolic fractions prepared from a variety of tissue and cell lysates (6) and may therefore shuttle between these compartments in response to phosphorylation. Whatever the consequences of phosphorylation it is intriguing that rap1GAP contains closely juxtaposed sites for cAMP-PK and p34<sup>cdc2</sup> kinase and that these two kinases typically have opposing effects on cell growth (24). However, a recent study indicated that cAMP-PK and p34<sup>cdc2</sup> may even associate with each other suggesting possible interdependent activities (25).

It is clear from this study that rap1GAP is significantly phosphorylated on serine residues both in vivo and in vitro. The major sites of phosphorylation are contained between methionine residues 445 and 545 in the complete 663-amino acid primary structure of rap1GAP. Interestingly, this region of rap1GAP also contains a duplicated amino acid sequence resulting from an alternative mRNA splicing event (6). Two distinct cDNAs coding for rap1GAP were found to differ by the inclusion of an additional 78 base pairs inserted in the 3' coding region of one of the clones (6). These additional base pairs code for a 26-amino acid sequence highly homologous to the sequence immediately following it and contained between residues 478-505 in the rap1GAP used in the present study (see Fig. 2). The repeated region present in the splicing variant encodes an additional putative cAMP-PK site, RGGS, but the p34<sup>cdc2</sup>-consensus site is not duplicated. Recombinant proteins expressed from either cDNA exhibit approximately equal specific activities toward p21<sup>r<sup>61</sup></sup> (6). This again suggests that the carboxyl-terminal portion of rap1GAP is not directly involved in the stimulation of p21<sup>r<sup>61</sup></sup> but rather may serve as a regulatory domain interfacing rap1GAP to the activation of serine kinases in the cell.

The cellular function of p21<sup>r<sup>61</sup></sup> is not understood. The protein has been shown to bind with high affinity to rasGAP (26, 27), and it has been suggested that this competition may account for its ability to revert the ras-transformed phenotype in 3T3 cells (28). The finding that rap1GAP is abundant in some myeloid cell types and is diminished following their differentiation suggests that the p21<sup>r<sup>61</sup></sup>rap1GAP system may participate in the control of cell growth (6). This is consistent with our results showing that rap1GAP is a good substrate for the cell cycle p34<sup>cdc2</sup> kinase. The phosphorylation of rap1GAP by cAMP-PK is intriguing considering that p21<sup>r<sup>61</sup></sup> itself is also a good substrate for this kinase. It would make sense that interacting proteins such as p21<sup>r<sup>61</sup></sup> and rap1GAP respond to the same second messenger systems. The identification of rap1GAP as a substrate for serine/threonine kinases and the identification of those kinases acting on it in vivo will help bring together the various components functioning in this signaling network.

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