THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 9, Issue of March 3, pp. 4805–4812, 1995
Printed in U.S.A.

Characterization of Rad, a New Member of Ras/GTPase Superfamily, and Its Regulation by a Unique GTPase-activating protein (GAP)-like Activity*

(Received for publication, August 3, 1994, and in revised form, December 2, 1994)

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We have recently identified a new member of the Ras/GTPase superfamily termed Rad which has unique sequence features and is overexpressed in the skeletal muscle of humans with type II diabetes (Reynet, C., and Kahn, C. R. (1993) *Science*, 262, 1441–1444). When expressed in bacteria as a glutathione *S*-transferase fusion protein, Rad bound [α-^{32}P]GTP quickly and saturably. Binding was specific for guanine nucleotides and displayed unique magnesium dependence such that both GTP and GDP binding were optimal at relatively high Mg^{2+} concentrations (1–10 mM). Rad had low intrinsic GTPase activity which was greatly enhanced by a GTPase-activating protein (GAP) activity present in various tissues and cell lines. Several known GAPs had no stimulatory effect toward Rad. Conversion of Ser to Asn at position 66 in Rad (equivalent to position 12 in Ras) resulted in a total loss of GTP binding. Mutation of Pro^{61} (equivalent to Gly^{12} in Ras) or Gln^{109} (equivalent to Gln^{61} in Ras) had no effect on Rad GTPase activity, whereas creation of a double mutation at these positions resulted in exceptionally high intrinsic GTPase activity. *In vitro*, Rad was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase (PK). Phosphopeptide mapping indicated two PKA phosphorylation sites near the C_{OOH} terminus. Rad also coprecipitated a serine/threonine kinase activity from extracts of various tissues and cell lines which catalyzed phosphorylation on Rad but was not inhibited by PKA inhibitor. Thus, Rad is a GTP-binding protein and a GTPase which has some structure/function similarities to Ras, but displays unique features. Rad may also be phosphorylated on serine/threonine residues by PKA and other kinases, as well as regulated by its own GAP which is present in many tissues and cell types.

The Ras superfamily of GTP-binding proteins has been implicated in a wide spectrum of cellular functions, including cell proliferation and differentiation (1), intracellular vesicular trafficking (2), and cytoskeletal control (3). Since the initial observation that microinjection of a neutralizing Ras antibody into *Xenopus* oocytes blocked insulin-stimulated maturation (4), evidence for the involvement of Ras-related proteins in the actions of insulin and other growth factors has accumulated at an increasingly rapid pace (5–9). Two members of Ras-related family of proteins, Rab3D (10) and Rab4 (11), have been implicated in the translocation of glucose transporters in response to insulin, and Rac has been implicated in insulin-stimulated membrane ruffling (12). By screening cDNA subtraction libraries, our laboratory has identified a transcript encoding a novel member of the Ras superfamily (13). This protein has unique sequence features compared to other Ras-like proteins and is overexpressed in skeletal muscle of humans with Type II diabetes as compared with non-diabetic and Type I diabetic humans. We termed this protein Rad for Ras-related protein associated with diabetes. Very recently, a second member of the Rad family, termed Gem, was identified by polymerase chain reaction display following activation of T-lymphocytes (14).

Rad and Gem have several unique structural features among the Ras/GTPase superfamily which might affect GTP binding or GTPase activity. Both Rad and Gem are longer than Ras due to NH_2- and COOH-terminal extensions; both lack the CAAX box at the COOH terminus which may affect their ability to undergo posttranslational modifications, such as geranyl-geranylation or farnesylation (15); and both contain a number of nonconserved sequence changes in regions G1, G2, and G3 which are known to be involved in GTP binding and GTP hydrolysis (see Fig. 1).[1]

All Ras-related proteins are GTPases. These GTPases cycle between a GTP-bound (presumably active) form and a GDP-bound (presumably inactive) form. This cycle is regulated by proteins that affect GDP dissociation, GTP association, or the rate of GTP hydrolysis (16). The former are represented by guanine nucleotide dissociation inhibitors and guanine nucleotide exchange factors, the best characterized of which is the mammalian homologue of the *Drosophila* Son-of-Sevenless protein (mSos). The latter is exemplified by GTPase-activating proteins (GAPs).[2] Both guanine nucleotide exchange factor and GAP activity may be controlled in response to extracellular stimuli (17). The role of Rad in the signaling events is still unknown. However, both Rad and Gem have marked sequence differences from any known Ras-related proteins in the effector (G2) domain thought to bind GAP. In the present study we

* This work was supported in part by National Institutes of Health Grant DK 45935 (to C. R. K.), Joslin's Diabetes and Endocrinology Research Center Grant DK 36836, an American Diabetes Association Mentor-based grant (to C. R. K.), and Juvenile Diabetes Foundation Postdoctoral Fellowship 392469 (to C. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Rad has 2 in-frame methionines which result in potential proteins of 269 and 308 amino acid residues. It is not known which of these is used *in vivo*. For the purpose of this study, the first amino acid residue of Rad is assigned to the second methionine (corresponding to the second in frame ATG) as was discussed previously (13). Therefore, residues 61, 66, 108, and 109 in Rad align with residues 12, 17, 60, and 61 in Ras, respectively.

[2] The abbreviations used are: GAP, GTPase-activating protein; PKA, protein kinase A; PKC, protein kinase C; GST, glutathione *S*-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GDPβS, guanosine 5′-*O*-(2-thiodiphosphate).

have expressed Rad protein, characterized its GTP binding and GTPase activity properties, and studied structure-function relationship by site-directed mutagenesis and the possible regulation by noncovalent and covalent modifications.

EXPERIMENTAL PROCEDURES

Construction of Rad and Expression in E. coli—Full-length rad starting at the second in frame ATG (from nucleotide 241 to 1050 in Ref. 13) was generated by the polymerase chain reaction using a plasmid containing a Rad cDNA, including untranslated regions as template. The resulting 1.2-kilobase pair DNA was subcloned into *Bam*HI-*Eco*RI-digested pGEX-2T vector (Pharmacia Biotech Inc.). The recombinant plasmid was transformed into XL1-blue cells (Stratagene), and expression of GST-Rad was induced with isopropyl-β-D-thiogalactopyranoside. The fusion protein was affinity-purified from the soluble fraction of cell extract with glutathione (GSH)-Sepharose beads (Pharmacia) (18). When Rad alone was needed, its GST fusion partner was cleaved off with thrombin (19).

Site-directed Mutagenesis—A transformer mutagenesis kit (Clontech) was used to generate various mutants of Rad. Briefly, synthetic oligonucleotide primers containing the mutated sequences were made and annealed to pGEX-2T-rad backbone along with a selection primer which switched a unique *Aat*II site on the vector to a unique *Sac*I site. The parental plasmids were linearized by extensive restriction selection and nascent mutagenic plasmids were transformed to a mismatch repair-deficient strain (BMH 71-18 mut S). The mutant plasmids were then transformed to XL1-blue cells for DNA sequencing and protein expression.

Cell Culture, Preparation of Cell Lysate, and Tissue Extracts—L6 rat myoblasts were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a water-saturated 10% CO_2, air atmosphere at 37 °C to near confluence. Cells were washed twice with cold phosphate-buffered saline and scraped into a sonication buffer containing 50 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM DTT, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 10 μM aprotinin. Cells were sonicated with a probe sonicator (Branson) and the particulate fraction removed by centrifugation at 1000 × *g* for 5 min followed by centrifugation at 100,000 × *g* for 60 min. The supernatant (cytosol) was aliquoted and stored at −80 °C after determination of protein concentration. For the preparation of tissue extracts, human skeletal muscle, and rat tissues were homogenized with a Polytron in the cold lysis buffer (sonication buffer plus 1.1% Triton X-100) and incubated at 4 °C for 90 min. The lysate was centrifuged at 100,000 × *g* for 60 min and the supernatant stored at −80 °C.

Guanine Nucleotide Binding—GTP binding to Rad was determined using a nitrocellulose filtration assay (13) or a GTP overlay assay (20). GST-Rad (20 pmol) was incubated in an exchange buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT, and 1 mg/ml bovine serum albumin) containing the indicated concentrations of $MgCl_2$ and 1.7 μM [α-^{32}P]GTP (3 μCi/sample; DuPont NEN) at 22 °C with end-to-end rotation. When needed, EDTA was added, and the concentrations of free Mg^{2+} calculated (21). At each time point, aliquots of 40 μl were withdrawn in duplicate and directly filtered through BA 85 nitrocellulose filters (Schleicher & Schuell) followed by washing with 12 ml of cold filtration buffer (50 mM Tris-HCl, pH 7.4, 0.1 mM DTT) containing the same concentrations of $MgCl_2$ as used in the binding assay. The radioactivity remaining on the filters was determined by scintillation counting. [^{3}H]GDP (3 μCi/20 pmol of GST-Rad; DuPont NEN) binding was carried out as described by Hall and Self (22) except that the reaction was carried out at 37 °C for 30 min in the absence or presence of various concentrations of Mg^{2+}, and the radioactivity retained on the nitrocellulose filters was determined as described above.

To assess the specificity of GTP binding to Rad, the fusion protein (5 μg/lane) was resolved on 10% SDS-PAGE followed by electrotransfer to nitrocellulose paper (Schleicher & Schuell). The blots were cut into strips, washed twice with overlay buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4/K_2HPO_4, and 0.3% Tween 20), and incubated in the overlay buffer containing 1 μCi/strip of [α-^{32}P]GTP in the absence or presence of various nucleotides at a concentration of 0.1 mM. Incubation was continued at room temperature for 60 min, after which the blots were washed twice for 10-min intervals with the same buffer supplemented with 20 mM $MgCl_2$. The blots were air-dried and exposed to X-Omat AR film (Kodak). The amount of GTP was quantitated by scanning densitometry (Molecular Dynamics).

GTPase Assay—GTPase activity was measured by thin layer chromatography (TLC) (23) using a hydrolysis buffer as described previously (24). GST-Rad-Sepharose beads (1 μg of protein) were loaded with [α-^{32}P]GTP (3 μCi) as described above at room temperature for 20 min. The beads were washed three times by microcentrifugation (with 30 μl of Sepharose 4B beads as carrier) with cold washing buffer (50 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml bovine serum albumin) and once with cold hydrolysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, and 1 mg/ml bovine serum albumin). The beads were resuspended in the hydrolysis buffer equilibrated to room temperature, and aliquots of 40 μl were transferred to microcentrifuge tubes containing 40 μl of buffer, different GAPs (p120GAP, NF1, and Rap-GAP were provided by Frank McCormick of Onynx; p190 and IQGAP1 were from Jeffery Settleman of Massachusetts General Hospital) or cell/tissue extracts and carrier beads. Hydrolysis was carried out at 22 °C and quenched by the addition of 1 ml of cold washing buffer followed immediately by washing twice with the same buffer. The bound nucleotides were then eluted with 20 μl of elution buffer consisting of 1% SDS and 20 mM EDTA at 65 °C for 5 min. The supernatant was collected by centrifugation. Equal amount of counts were loaded onto polyethyleneimine cellulose thin layer chromatography plates (EM Science), and nucleotides were resolved in 0.75 M K_2PO_4, pH 3.4. TLC plates were exposed to x-ray film and the relative amounts of GTP and GDP quantitated by scanning densitometry (Molecular Dynamics).

Co-precipitation and in Vitro Phosphorylation—Tissue or cell extracts (200 μg/assay) obtained as described above were precleared with 500 μg of GST-Sepharose beads five times by incubation at 4 °C for periods of 30 min. The precleared extract was then incubated with GST-Rad-Sepharose beads (20 μg/assay) in the presence of 0.1 mM GTPγS or GDPβS at 4 °C for 90 min followed by washing five times with the extraction buffer containing 0.1 mM GTP or GDP. To assess phosphorylation, the beads were resuspended in the same buffer containing [γ-^{32}P]ATP (10 μCi/assay; DuPont NEN) and incubated at 30 °C for 15 min. The beads were then washed twice, and phospho-Rad was resolved by 10% SDS-PAGE. The gel was stained, destained, dried, and exposed to x-ray film.

GST-Rad was also phosphorylated *in vitro* with the catalytic subunit of protein kinase A (PKA, Sigma; 20 μg of fusion protein/25 units of PKA/assay) in a kinase buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT), and samples were resolved by SDS-PAGE and autoradiography as described above. When Rad was to be separated from its fusion partner GST, the phosphorylated GST-Rad beads were incubated in a cleavage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$) containing 0.2 μg thrombin (Sigma) at room temperature for 60 min (19).

Phosphopeptide Mapping—Two potential PKA-mediated phosphorylation sites are located near the COOH terminus of Rad (Ser^{218} and Ser^{234}). Rad also possesses several potential V8 protease cleavage sites. Cleavage at Glu^{206}, Glu^{220}, and Glu^{230} can produce two peptides bearing potential PKA phosphorylation sites, with predicted molecular weight of 1.6 and 4.1 kDa, respectively. V8 protease (Boehringer Mannheim) was used to cleave between these two sites. GST-Rad-Sepharose beads (10 μg) were incubated in kinase buffer in the presence of 25 units of PKA and 10 μCi of [γ-^{32}P]ATP at 30 °C for 15 min. The beads were washed four times with the same buffer and resuspended in 30 μl of V8 cleavage buffer (250 μl of SDS-PAGE stacking gel buffer, pH 6.8, 100 μl of 10% SDS, 100 μl of glycerol, 2 μl of 0.5 M EDTA, 3 μl of 2-mercaptoethanol, and 630 μl of H_2O) followed by a 2-min boiling to denature the protein. After brief centrifugation, V8 protease was added to the supernatant, and the cleavage was carried out at 37 °C for 1 h (25). The phosphopeptides were resolved in 15% SDS-PAGE. The gel was stained, destained, dried, and exposed to x-ray film for 1–5 min.

RESULTS

Rad Is a GTP-binding Protein—As was predicted from its primary sequence, Rad bound [α-^{32}P]GTP in a rapid, saturable, and time-dependent fashion. GTP binding to GST-Rad was rapid with half-maximal binding at 1 min and maximal by 10 min at 22 °C (Fig. 2*A*); GST alone had no guanine nucleotide binding activity (data not shown). Binding of both GTP and GDP to Rad was Mg^{2+}-dependent (Fig. 2, *A* and *B*). In contrast to $p21^{N\text{-}ras}$ which shows increased exchange rates at low Mg^{2+} concentration (22), GTP and GDP binding to Rad were minimal when the calculated Mg^{2+} was 0.5 μM, but increased at higher Mg^{2+} concentrations, reaching maxima at 1 and 10 mM of $MgCl_2$, respectively (Fig. 2*B*). Binding to Rad could also be demonstrated using a GTP overlay assay. Binding of [α-^{32}P]GTP was completely blocked by 0.1 mM of GTP, GTPγS,

FIG. 1. **Comparison of amino acid sequences of Rad, Gem, and N-Ras.** Predicted amino acid sequences of Rad, Gem, and Ras are aligned and numbered with the second in-frame methionine of Rad as position one and the first in frame methionine of Rad as position −39. The five G domains are *shaded* and the variations in positions 61 and 108 are marked. *Dashes* indicate gaps inserted for optimal alignment of the sequences.

GDP, and GDPβS, whereas pyrimidine nucleotides did not compete to any extent (Fig. 2*C*). ATP also showed slight inhibition of GTP binding to Rad at high concentrations (Fig. 2*D*).

Rad Is a GTPase—To assess GTPase activity, Rad was loaded with [α-^{32}P]GTP, free nucleotides were washed away, and bound GTP was subjected to hydrolysis in the presence of 10 mM $MgCl_2$. At each time point, the reaction was stopped, and bound nucleotides were eluted and analyzed by TLC. Under these conditions, Rad itself produced slow hydrolysis of GTP to GDP, suggesting a low intrinsic GTPase activity. Addition of cytosol from L6 myoblasts greatly accelerated GTP hydrolysis by Rad (Fig. 3*A*). In fact, almost all the bound GTP was converted to GDP by 20-min incubation at room temperature in the presence of 40 μg of total cytosolic protein. A similar stimulation was observed using both cytosolic and membrane fractions from 3T3-L1 cells, 3T3-F442A cells, and Chinese hamster ovary cells (data not shown). Denaturation of the L6 cell extract at 100 °C for 2 min completely inactivated this GAP activity, suggesting that this activity was due to a protein in the cell extract that stimulated Rad GTPase (Fig. 3*B*). A panel of known GAPs, including Ras-GAP, NF1, p190, Rap-GAP, and IQGAP1 (26), were tested for their abilities to stimulate Rad-GTPase activity. None of these proteins had noticeable effect on the GTPase activity of Rad (Fig. 3*B*). Rad-GAP activity was present in every rat tissue tested, although the maximal stimulation with skeletal muscle was somewhat lower than that of other tissues (Fig. 3*C*). Stimulation of Rad GTPase activity by different rat tissues was dose-dependent (Fig. 3*D*).

Analysis of Rad by in Vitro Mutagenesis—Aligning the sequences of Rad and Gem with Ras (Fig. 1) reveals a number of potentially interesting, nonconservative sequence substitutions which could affect GTP binding and GTPase properties. First, the highly conserved Gly^{12} in Ras is replaced by Pro^{61} in Rad and Gln^{84} in H-Gem. Also both Rad and Gem possess a Glu at the position corresponding to the highly conserved Gly^{60} in Ras. On the other hand, certain functionally important residues are conserved, for example, Ser^{66} and Gln^{109} in Rad (corresponding to Ser^{17} and Gln^{61} in Ras, respectively). Mutation of Ser^{17} to Asn creates a dominant negative Ras, whereas Gln^{61} is often found to be mutated, alone or together with Val^{12}, in transforming forms of Ras (27).

To better understand the structure-function relationship of Rad, we performed *in vitro* site-directed mutagenesis. Changes in activity of the various mutants with regard to GTP binding and GTP hydrolysis are summarized in Table I. As with Ras itself, a point mutation of Ser^{66} to Asn^{66} in Rad completely abolished its ability to bind GTP (Fig. 4*B*), indicating the critical role of this residue in GTP binding or overall structure of the protein. Point mutation of Pro^{61} to Gly (the amino acid found in Ras) slightly decreased GTP binding, whereas modification of this residue to Val (a transforming mutation in Ras) did not result in significant

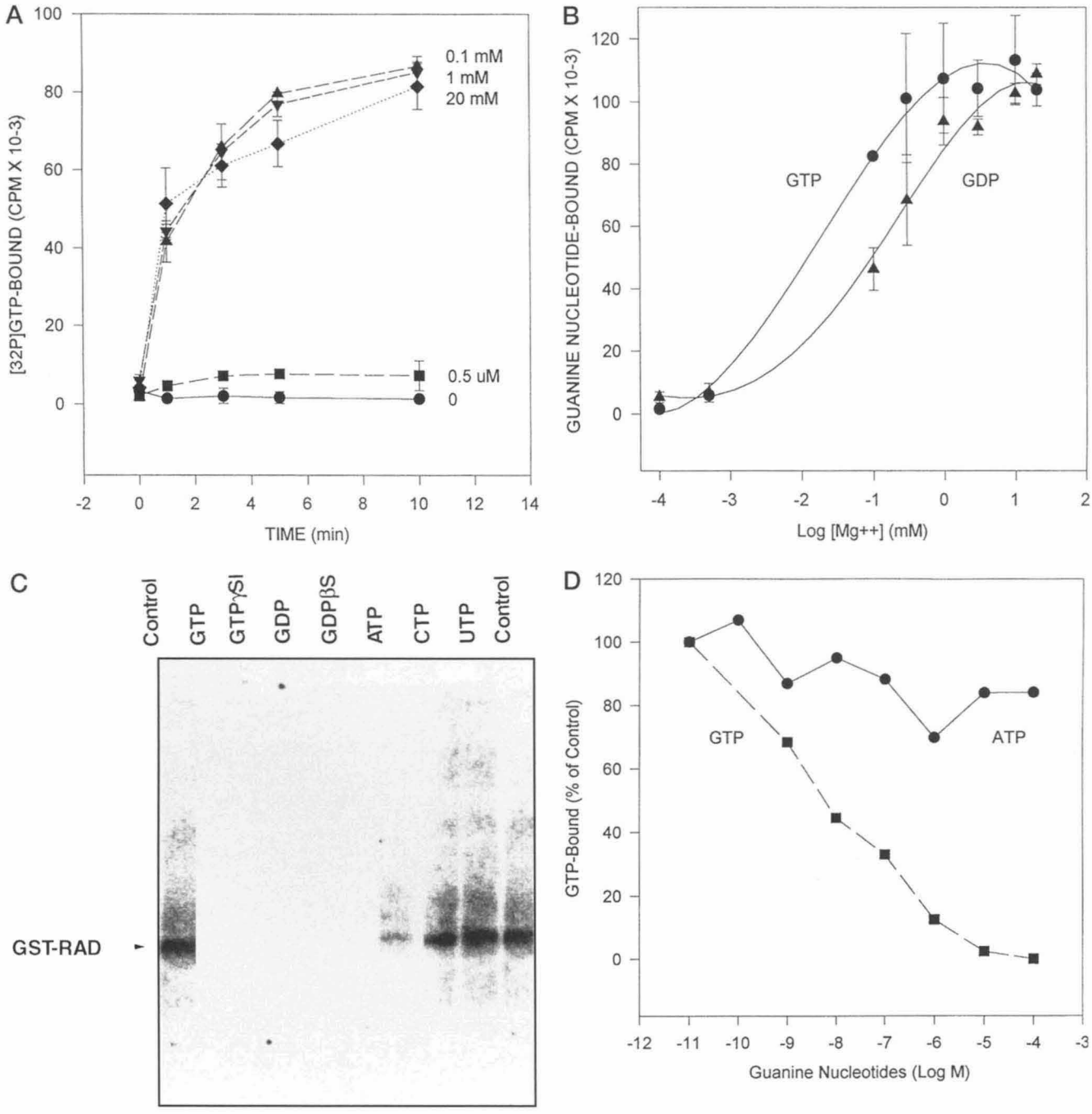

FIG. 2. **Rad is a GTP-binding protein.** *A*, GST-Rad (1 μg) was incubated with [α-^{32}P]GTP (3 μCi/1.7 μM) in the absence or presence of various concentrations of Mg^{2+} at 22 °C for the indicated time. The calculated free Mg^{2+} concentrations (mM) were: ●, 0; ■, 0.0005; ▲, 0.1; ▼, 1; ♦, 20 and were produced by appropriate mixture of $MgCl_2$ and EDTA (21). *B*, GST-Rad (1 μg) was incubated with [α-^{32}P]GTP (3 μCi/1.7 μM) or [^{3}H]GDP (3 μCi/2.2 μM) in the absence or presence of various concentrations of $MgCl_2$ at 22 °C for 10 min or 37 °C for 30 min. The symbols represent GTP binding (●) and GDP binding (▲). *C*, GST-Rad (5 μg) was resolved by 10% SDS-PAGE, transferred to nitrocellulose filters, and probed with [α-^{32}P]GTP (1 μCi/lane) in the absence or presence of 0.1 mM of indicated nucleotides as described under "Experimental Procedures." *D*, competition studies were performed as described for *C* with individual nitrocellulose strips incubated with various concentrations of GTP (■) or ATP (●). Data in *A* and *B* are the mean from two or three separate experiments performed in triplicate (*A*) or duplicate (*B*). The data in *C* and *D* are the representative of three separate blots and average of two separate quantitative determinations.

changes in either GTP binding or hydrolysis. Mutation of Gln^{109} to His slightly increased GTP binding, but was without effect on GTP hydrolysis. However, a double mutant, P61V/Q109H, showed extremely high intrinsic GTPase activity, which was further stimulated to near complete GTP hydrolysis within 3 min upon addition of as low as 10 μg of total rat liver cytosolic proteins.

Rad as a Phosphoprotein—Examination of the amino acid sequence of Rad indicated that Rad possesses one potential ATP-binding site near amino terminus (G^{23}TGTQG^{28}) and two potential phosphorylation sites for PKA (28) near the carboxyl-terminal, RRD**S**^{218} and RRE**S**^{234}. Rad also has at least 10 potential sites for protein kinase C (PKC)-mediated phosphorylation throughout its primary sequence. To test whether Rad is a substrate for PKA or PKC, GST-Rad was subjected to *in vitro* phosphorylation by the catalytic subunit of PKA or a constitutively active PKC (provided by Heimo Riedel of Joslin Diabetes Center) and followed by thrombin cleavage of Rad

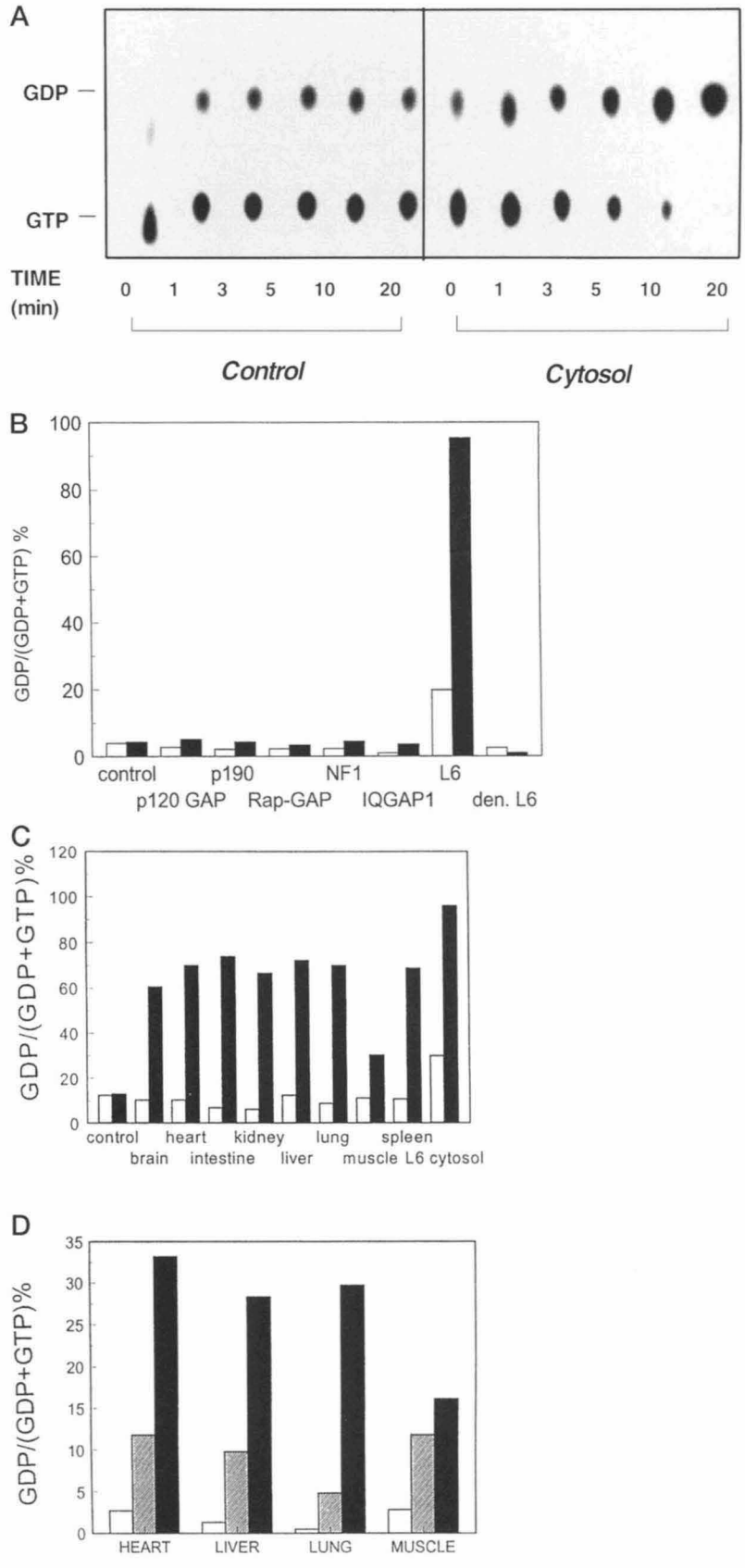

FIG. 3. **Rad is a GTPase.** GST-Rad (1 μg) bound to GSH-Sepharose beads was loaded with [α-^{32}P]GTP (3 μCi) at 22 °C for 10 min. The beads were washed in cold washing buffer, and GTP hydrolysis preceded at 22 °C for the indicated times (*A–C*) or 10 min (*D*) in the absence or presence of various GAPs. The beads were washed, bound nucleotides were eluted and resolved by polyethyleneimine cellulose thin layer chromatography as described under "Experimental Procedures." *A* shows a typical autoradiogram representative of three separate experiments. For *B–D*, the autoradiograms were quantified by scanning densitometer and the data expressed as percent of GDP in the total bound guanine nucleotides for two or three separate experiments.

from GST. Rad was phosphorylated in the presence, but not absence, of PKA (Fig. 5*A*); however, it failed to undergo *in vitro* phosphorylation in the presence of constitutively active PKC (data not shown). To confirm the sites of PKA phosphorylation, the phosphorylated Rad was digested with V8 protease which cleaves between the two potential PKA phosphorylation sites. If the cleavage was complete, a phosphopeptide of 1.6-kDa bearing Ser^{218} and another of 4.1 kDa with Ser^{234} should be resolved. After extensive cleavage, two phosphopeptides were observed in gel electrophoresis; one migrated just above the dye

TABLE I
Functional comparison of wild type and mutant Rad

All the bacterial-expressed Rad mutants were prepared simultaneously with the wild type Rad to minimize variation in preparation. GTP binding and GTPase activity were measured as described in the legends of Figs. 2 and 3. Each mutant was assayed with wild type Rad as reference. The data were expressed as the changes in GTP binding and GTP hydrolyzing activities normalized to the percentage of that of wild type Rad and were the means of two to four separate determinations. ND, not determined.

Rad/mutants	GTP binding	Basal GTP hydrolysis
	% wild type	
Wild type	100	100
P61G	40–60	100
P61V	40–60	100
S66N	0	ND
E108G	100	100
Q109H	120–140	100
P61V/Q109H	ND	400 –700

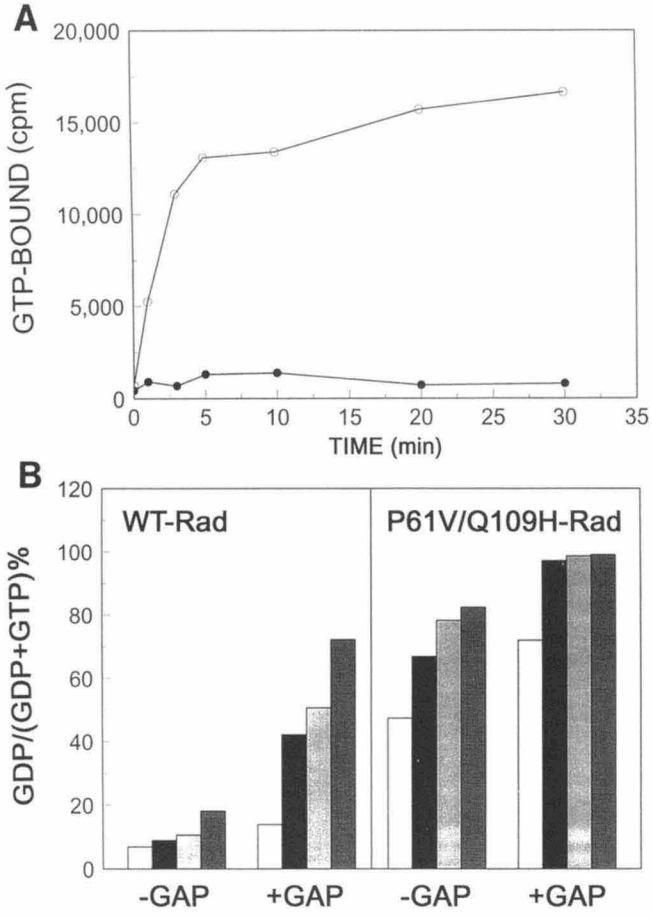

FIG. 4. **Mutational analysis of Rad.** Point mutations were made using wild type pGEX-2T-rad as template. Each mutant was confirmed by DNA sequencing. *A*, [α-^{32}P]GTP binding to wild type (○) and S66N (●) Rad. Binding was done as described in the legend to Fig. 2, except that 0.25 μg/assay of Rad protein was used. *B*, [α-^{32}P]GTP hydrolysis by wild type (*WT*) and P61V/Q109H Rad. GTP hydrolysis was assessed as described in the legend to Fig. 2. Data are the means of three (*A*, triplicate) or two (*B*, single data points) separate experiments.

front on a 15% SDS-PAGE, whereas the other migrated between the 6.5- and 3.4-kDa molecular markers (Fig. 5*B*). This result suggested that both sites were phosphorylated by PKA.

Evidence for a Rad-associated Protein Kinase—To determine if Rad might form a complex through specific protein-protein interaction with a kinase capable of phosphorylating Rad, GST-Rad-Sepharose beads were used to precipitate the extract from human skeletal muscle. After extensive washing, Rad was found to precipitate a kinase activity which catalyzed *in vitro* phosphorylation of Rad in the presence of [γ-^{32}P]ATP (Fig. 5*C*). A similar result was obtained with the lysate from L6 myoblast cells (Fig. 5*D*). Rad precipitated this kinase activity from all the cell lines and tissues tested in both the GTP- and GDP-bound states (data not shown). Phosphorylation was more prominent in the presence of GDP than GTP. This most likely reflects a preferential competition of GTP with [γ-^{32}P]ATP during the phosphorylation. Phosphorylation by this endogenous kinase appears to be on serine/threonine residues, since anti-phosphotyrosine antibodies did not recognize phosphorylated Rad (data not shown). At present, the nature of the Rad-associated kinase from various tissues and cell lines is unknown. However, it is probably not PKA, since it was not stimulated by forskolin or inhibited by protein kinase A inhibitor (data not shown).

DISCUSSION

We have expressed Rad, a novel Ras-related protein overexpressed in skeletal muscle of humans with type II diabetes, characterized its nucleotide binding and enzymatic activity and regulation by covalent and noncovalent interactions. We find that Rad is a GTP-binding protein and GTPase-regulatable by a specific GAP-like activity and may also be subjected to serine/threonine phosphorylation.

Binding of GTP to Rad is fast and saturable, a characteristic shared by all Ras-like proteins. For example, like human Ha-Ras p21 (29) and other small G proteins (30), Rad bound specifically [α-^{32}P]GTP after transfer to nitrocellulose filters, indicating that in a denatured state Rad still retains the ability for ligand binding. However, Rad displays several features that are clearly different from the other members of Ras family. Thus, N-Ras has a low and linear rate of guanine nucleotide binding at high (5 mM) magnesium concentrations and increases its exchange rate at low (0.5 μM) magnesium concentrations (22). By contrast, Rad binds minimal amounts of GTP or GDP at 0.5 μM Mg^{2+}, but increases binding significantly at higher magnesium concentrations. Considering that intracellular [Mg^{2+}] is about 30 mM (31), with [GTP] far exceeding [GDP] (32), it is conceivable that Rad may be in a state of high activity in the normal magnesium concentration in cells.

The unique pattern of Mg^{2+} dependence may result from the sequence variation of Rad in the G3 domain (which is known to be important for interaction with Mg^{2+}/GTP (33)), since the highly conserved Ala^{59} and Gly^{60} residues in most other Ras proteins are replaced with Trp^{107} and Glu^{108} residues in Rad. However, when these two nonconserved amino acid residues are changed to be identical to those in Ras, the "Ras-like" binding characteristics (with regard to Mg^{2+} dependence) are not restored. On the other hand, like the mutant $Ras^{Asn^{17}}$, Rad completely loses the GTP binding activity when Asn replaces the Ser residue at position 66. Because a Ras-like protein is presumably inactive at its GDP-bound form, this loss-of-function mutant may provide a potentially dominant negative form and may serve as a powerful tool for further studies of biological roles of Rad *in vivo*. Furthermore, whereas Rad tolerated the single mutation at either position 61 or 109, the double mutant, P61V/Q109H, displays very high intrinsic GTPase activity approaching that of GAP-stimulated GTP hydrolysis. It is possible that this mutant protein assumes a conformation that resembles one which has been induced to confer by a GAP, although this activity can be further stimulated to near maximum by rat liver cytosolic proteins. Further comparative structural studies (*e.g.* x-ray crystallography) of this unique mutant and the wild type Rad may help revealing the conformation requirements for GTPases to hydrolyze GTP.

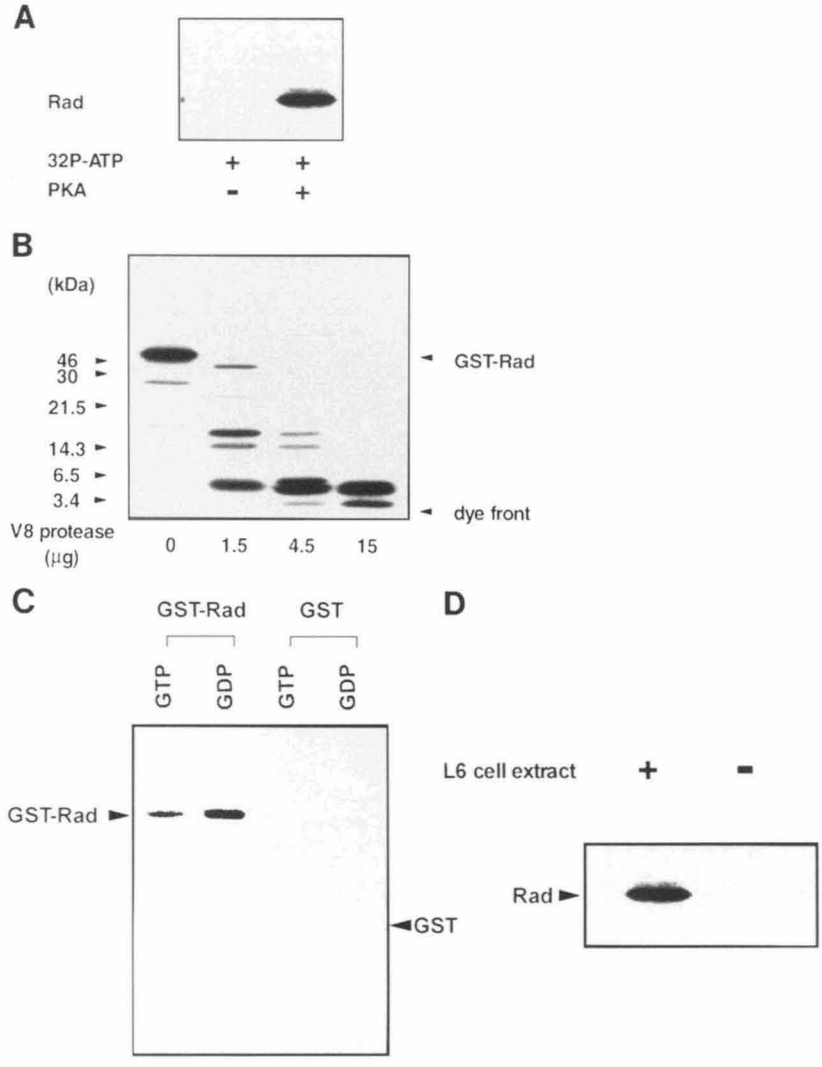

FIG. 5. **Rad as a phosphoprotein.** *A*, GST-Rad (20 μg) immobilized on GSH-Sepharose beads was incubated with the catalytic subunit of PKA (25 units) and [γ-^{32}P]ATP (10 μCi) at 30 °C for 15 min. The beads were washed, and GST was cleaved from Rad by thrombin (0.2 μg) at 22 °C for 60 min. The products, including phospho-Rad, were then resolved by 10% SDS-PAGE. *B*, GST-Rad was phosphorylated as described above and digested with V8 protease at the indicated concentrations at 37 °C for 1 h after heat denaturation followed by SDS-PAGE on 15% gel to resolve the phosphopeptides. *C* and *D*, GST-Rad (20 μg) immobilized on GSH-Sepharose beads was incubated with human skeletal muscle or L6 cell cytosolic extracts precleared with GST-Sepharose beads at 4 °C for 90 min. The beads were then subjected to extensive washings as described under "Experimental Procedures" and incubated with [γ-^{32}P]ATP (10 μCi) at 30 °C for 15 min. Phospho-Rad was detected either by direct 10% SDS-PAGE (*C*) or cleaved with thrombin followed by electrophoresis (*D*). The data are the representative of two to five separate experiments.

GAPs interact with GTPases and greatly accelerate the rate of GTP hydrolysis. Most GAPs are relatively specific in that they exert their effects only on certain subfamilies or subtypes of GTPases. Rad has low intrinsic GTPase activity, and thus, it is reasonable to suggest that to efficiently "turn off" Rad *in vivo*, a GAP must exist. However, Rad is unresponsive to p120 GAP and NF1 which act on Ras (34), p190 which acts on Rac and Rho GTPases (24), Rap-GAP (35), and the recently cloned IQGAP1 (26). Specific Rad-GAP activity, however, is present in all mammalian tissue and cell lines studied. The fact that rat skeletal muscle extract does not show a linear dependence of GAP activity suggests co-expression of other factors that can also affect the GTPase activity of Rad. The ubiquitous distribution of Rad-GAP activity may suggest a role for Rad or Rad-related proteins such as Gem in a variety of cells. At present, the exact nature of Rad-GAP is not known. Preliminary studies have shown that it is a heat-sensitive protein, highly hydrophilic and positively charged at pH 6.0, and elutes in a molecular mass range between 60 and 100 kilodaltons on gel filtration chromatography (data not shown). Purification and characterization studies are under way to identify this GAP.

Although phosphorylation of Ras-like proteins does not regulate their GTP binding and GTPase activities, phosphorylation may affect cellular localization. For example, Rap1B in platelets is translocated from membranes to the cytoplasm when it is phosphorylated by PKA in response to prostaglandin E_1 and dibutyryl cAMP stimulation. This also increases the sensitivity of Rap1B to its guanine nucleotide exchange factor (36). Rab4 is serine-phosphorylated by cdc2 kinase in mitotic cells, and this enables Rab4 to be released into cytosol (37). Subsequent dephosphorylation allows reassociation of soluble Rab4 with membranes upon exit of cells from mitosis. Rad may also be regulated by phosphorylation. Indeed, Rad is phosphorylated *in vitro* by PKA near its carboxyl terminus. The function of this phosphorylation is unknown, but phosphorylation did not affect Rad's GTP binding or basal and stimulated GTPase activity (data not shown), consistent with the effects of phosphorylation on Rap1A, Rap1B, and Rab4. Rad is also phosphorylated by a kinase that appears to be constitutively associated with Rad which we have tentatively termed Rad-associated kinase. Rad-associated kinase is a serine/threonine protein kinase, but is clearly different from PKA, since it is not stimulated by forskolin or inhibited by PKA inhibitor. The identification of this kinase activity may help to elucidate the signal transduction pathway in which Rad is involved.

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