Effects of phosphorylation on function of the Rad GTPase

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Rad, Gem and Kir possess unique structural features in comparison with other Ras-like GTPases, including a C-terminal 31-residue extension that lacks typical prenylation motifs. We have recently shown that Rad and Gem bind calmodulin in a Ca^{2+}-dependent manner via this C-terminal extension, involving residues 278–297 in human Rad. This domain also contains several consensus sites for serine phosphorylation, and Rad is complexed with calmodulin-dependent protein kinase II (CaMKII) in C2C12 cells. Here we show that Rad serves as a substrate for phosphorylation by CaMKII, cAMP-dependent protein kinase (PKA), protein kinase C (PKC) and casein kinase II (CKII) with stoichiometries *in vitro* of 0.2–1.3 mol of phosphate/mol of Rad. By deletion and point mutation analysis we show that phosphorylation by CaMKII and PKA occurs on a single serine residue at position 273, whereas PKC and CKII phosphorylate multiple C-terminal serine residues, including Ser^{214}, Ser^{257}, Ser^{273}, Ser^{290} and Ser^{299}. Incubation of Rad with PKA decreases GTP binding by 60–70 %, but this effect seems to be independent of phosphorylation, as it is observed with the $Ser^{273} \rightarrow Ala$ mutant of Rad containing a mutation at the site of PKA phosphorylation. The remainder of the serine kinases have no effect on Rad GTP binding, intrinsic GTP hydrolysis or GTP hydrolysis stimulated by the putative tumour metastasis suppressor nm23. However, phosphorylation of Rad by PKC and CKII abolishes the interaction of Rad with calmodulin. These findings suggest that the binding of Rad to calmodulin, as well as its ability to bind GTP, might be regulated by the activation of several serine kinases.

INTRODUCTION

Rad, Gem and Kir form a unique subfamily of Ras-related GTPases [1–3]. Characteristics of these molecules are extended N- and C-termini compared with Ras and a lack of the prenylation motifs typical of Ras. Rad has been implicated as an inhibitor of glucose transport in cultured muscle and fat cells and binds skeletal muscle β-tropomyosin and calmodulin (CaM) in a Ca^{2+}-dependent manner both *in vitro* and *in vivo* [4–6]. Rad activity is regulated in a novel bidirectional mechanism by the putative tumour metastasis suppressor nm23, which promotes both GTP hydrolysis and GTP reloading of Rad (J. Zhu, J. D. Kantor, C. J. Rhodes, B. R. Zetter, J. S. Moyers and C. R. Kahn, unpublished work). The binding of Rad to CaM occurs via the extended C-terminus of Rad, especially residues 278–297, which is predicted to form an amphipathic α-helix. Deletion of this region abolishes CaM binding and results in the disruption of Rad from cytoskeletal and membrane compartments in C2C12 myoblasts [6]. In addition to CaM, Rad also binds calmodulin-dependent protein kinase II (CaMKII) and is a substrate for phosphorylation by CaMKII and cAMP-dependent kinase (PKA) *in vitro* [6,7].

Few small GTP-binding proteins have been reported to be phosphorylated and, with the exception of Rab4, the regulatory role of these phosphorylations remains unknown. K-ras is phosphorylated *in vitro* and *in vivo* by protein kinase C (PKC) and PKA [8,9], whereas the Ras-like small GTP-binding protein smg p21 is phosphorylated by PKA and cGMP-dependent protein kinase *in vitro* and in prostaglandin E1-treated human platelets [10,11]. Phosphorylation, possibly by p34 cdc2 kinase, has been suggested to have a role in the reversible translocation of Rab4 during mitosis [12]. Rap1B is a substrate for PKA in human neutrophils and for a neuronal Ca^{2+}/CaM kinase *in vitro* [13,14]. In addition, Gem has been reported to be phosphorylated on tyrosine residues in mitogen-induced 3T3 cells [2].

The C-terminus of Rad contains several consensus sites for phosphorylation by CaMKII, PKC, PKA and casein kinase II (CKII) [1]. Some of these sites reside near the region of CaM binding and have the potential to affect the accessibility of this domain by introducing negative charges in the positively charged environment that binds CaM. In this study we show that Rad protein serves as an efficient substrate for CaMKII, PKA, PKC and CKII *in vitro*. Phosphorylation occurs on serine residues near the C-terminus of Rad; in PKC and CKII it abolishes CaM binding. Incubation of Rad with PKA also produces a unique decrease in GTP binding. These findings suggest that serine kinases have a role in the interaction of Rad with the CaM system and in its GTP binding.

MATERIALS AND METHODS

Construction of Rad mutants

The C-terminal deletion mutants terminating at Rad residues 249 and 297 (termed C249 and C297), were prepared as described [6]. Point mutations S214A, S257A, S273A and S290A, in which Ser^{214}, Ser^{257}, Ser^{273} and Ser^{290} have been converted to alanine residues, were generated by PCR with wild-type (WT) human Rad cDNA as a template. For the conversion of Ser^{214} to alanine, overlapping mutagenesis primers were used that contained a single base change at residue 763 to convert the serine codon (TCG) to an alanine (GCG) [1]. Other mutagenesis primers contained two base changes to convert the Ser^{257}, Ser^{273} and Ser^{290} codons (AGC) to alanine (GCC). The mutagenesis primers were used in combination with primers spanning an internal *Pfl*M1 site upstream of the sites of mutation or an *Eco*R1 site in the 3′ non-coding region of Rad in two-step PCR reactions as described [15]. Reaction products were cloned into the *Pfl*M1–*Eco*R1 sites of the pGEX-WT Rad (residues 39–308) vector to replace the C-terminal sequences of the WT construct.

Abbreviations used: CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; CKII, casein kinase II; DTT, dithiothreitol; GST, glutathione S-transferase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; WT, wild-type.

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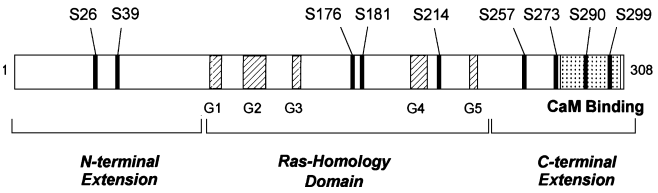

Figure 1 Potential phosphorylation sites in Rad

The human Rad sequence contains five potential CaMKII sites [RXXS/T], five potential PKA sites [RXS/T] or [RR/KXS/T], nine potential PKC sites [S/TXK/R], [K/RXXS/T] or [K/RXS/T] and one potential CKII site [S/TXXE].

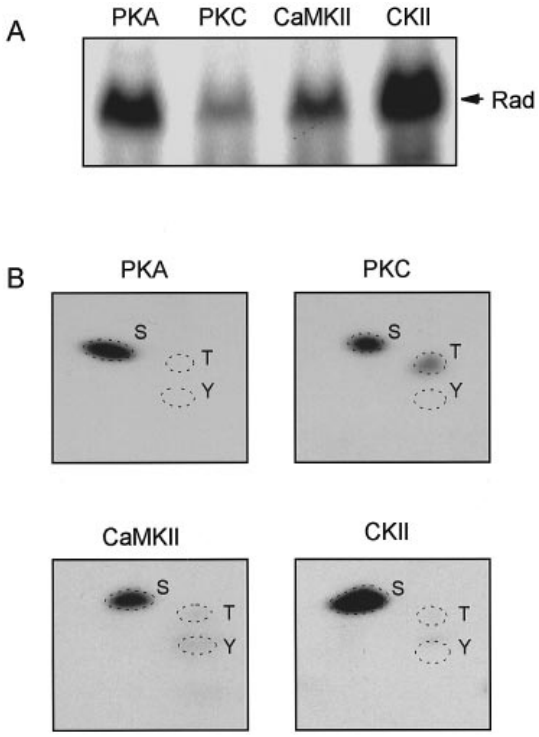

Figure 2 Rad is phosphorylated by CaMKII, PKA, PKC and CKII *in vitro*

GST–Rad (5 μg) was incubated in a kinase reaction *in vitro* containing [γ-^{32}P]ATP and CaMKII (100 units), PKA (0.05 unit), PKC (0.02 unit) or CKII (50 units), as described in the Materials and methods section. After incubation for 45 min at 30 °C, reaction products were subjected to SDS/PAGE and detected by autoradiography (**A**). The migration of GST–Rad is indicated. Gel slices containing phosphorylated Rad were excised and the protein was eluted and subjected to phosphoamino acid analysis as described in the Materials and methods section (**B**). The migration of the phosphoamino acid standards phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) are indicated.

All constructs were confirmed by DNA sequence analysis. Purified recombinant WT and mutant glutathione S-transferase (GST)–Rad proteins were prepared as described previously for Rad [7].

Kinase reactions *in vitro*

WT or mutant GST–Rad (5 μg) was incubated for various periods at 30 °C with the indicated concentrations of kinase in a 30 μl reaction in the presence of 100 μM ATP and [γ^{32}P]ATP (10 μCi per assay; DuPont–NEN). Phosphorylation by a truncated version of CaMKII (New England Biolabs) was performed in 20 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$/0.5 mM dithiothreitol (DTT)/0.1 mM EDTA supplemented with 2.4 μM CaM and 2 mM $CaCl_2$. The reaction buffer for phosphorylation by the catalytic subunit of PKA (New England Biolabs) was 50 mM Tris/HCl (pH 7.5)/10 mM $MgCl_2$. Phosphorylation by recombinant CKII (New England Biolabs) was performed in 20 mM Tris/HCl (pH 7.5)/50 mM KCl/10 mM $MgCl_2$. Incubations with PKC (a mixture of isoforms, primarily α and β; Promega) were performed in 20 mM Hepes (pH 7.4)/10 mM $MgCl_2$/1 mM $CaCl_2$ with 200 μg/ml phosphatidylserine and 10 μg/ml diolein. The samples were resolved by SDS/PAGE and detected by autoradiography. For stoichiometry determinations, Rad bands were excised; the counts incorporated were determined by Čerenkov counting.

Phosphoamino acid analysis

Two-dimensional phosphoamino acid analysis was performed by the method of Boyle et al. [16]. Phosphorylated Rad was eluted from gel slices, precipitated with trichloroacetic acid and subjected to treatment with 6 M HCl for 2 h at 90 °C. Products were freeze-dried and subjected to electrophoresis with phosphoamino acid standards on thin-layer cellulose plates (EM Science) with pH 1.9 buffer [formic acid/acetic acid/water (50:156:1794, by vol.)] in the first dimension and pH 3.5 buffer [acetic acid: pyridine:water (100:10:1890, by vol.)] in the second dimension. Standards were detected with ninhydrin and plates were analysed by autoradiography.

Western immunoblotting

Immunoblotting for Rad was performed with an anti-Rad polyclonal antibody (1:1000 dilution) followed by detection with ^{125}I-labelled protein A (DuPont–NEN) as described [5].

CaM–Sepharose binding

For binding to CaM–Sepharose or Sepharose 4B, 500 ng of GST–Rad was incubated in buffer [1% (w/v) Triton X-100/25 mM Tris/HCl (pH 7.4)/150 mM NaCl/1 mM DTT/-1 mM $MgCl_2$/1 mM PMSF/10 μg/ml aprotinin/10 μg/ml leupeptin] with 20 ml of a 50% (w/v) slurry of CaM–Sepharose (final concentration approx. 6 mM; Pharmacia) or Sepharose 4B (Pharmacia) which had previously been washed three times in buffer. After rotation for 2 h at 4 °C, beads were washed three times and bound proteins were analysed by SDS/PAGE and Western immunoblotting for Rad.

GTP binding

GTP binding to Rad was determined with a nitrocellulose filtration assay as described [7]. GST–Rad (20 pmol per reaction) was incubated with 100 μM ATP in kinase reactions *in vitro* with CaMKII (100 units), PKA (0.05 unit), PKC (0.02 unit) or CKII (50 units) as described above. Exchange buffer (500 μl) [50 mM Tris/HCl (pH 7.4)/1 mM DTT/1 mM $MgCl_2$/1 mg/ml BSA] containing [^{3}H]GTP (3 μCi per sample; DuPont–NEN) was added, and GTP binding proceeded at room temperature. At each time point, 40 μl was withdrawn in duplicate and applied to BA 85 nitrocellulose filters (Schleicher & Schuell) followed by washing with 10 ml of ice-cold filtration buffer [50 mM Tris/HCl (pH 7.4)/0.1 mM DTT/1 mM $MgCl_2$]. The radioactivity remaining on the filters was determined by scintillation counting.

GTPase activity

GTPase activity was measured by TLC [7]. GST–Rad–Sepharose beads (1 μg of protein) were incubated with 100 μM ATP in kinase reactions *in vitro* with CaMKII, PKA, PKC or CKII as described for GTP binding assays. Beads were washed three times with ice-cold loading buffer [50 mM Tris/HCl (pH 7.4)/-1 mM $MgCl_2$/1 mM DTT/1 mg/ml BSA] before being loaded with [α-^{32}P]GTP (3 μCi; DuPont–NEN) for 5 min at room temperature. Beads were washed three times in loading buffer, and GTP hydrolysis was performed for 5 min at room temperature in the presence or absence of 0.5 μg of GST–nm23 [7]. Equal radioactive counts of reaction products were subjected to TLC and analysed with a Molecular Dynamics PhosphorImager.

RESULTS

Rad is phosphorylated by CaMKII, PKA, PKC and CKII *in vitro*

Rad contains several consensus sites for phosphorylation, including five potential CaMKII sites, five potential PKA sites, nine potential PKC sites and one potential CKII site (Figure 1). We have previously shown that Rad serves as a substrate for CaMKII and PKA [6,7]. To explore Rad phosphorylation further, GST–Rad was incubated with CaMKII, PKA, PKC or CKII *in vitro*, as described in the Materials and methods section, and the reaction products were analysed by SDS/PAGE. All four serine kinases produced easily detectable Rad phosphorylation (Figure 2A). Determination of the quantity of ATP incorporated into Rad from counting of gel slices indicated stoichiometries of phosphorylation (means $\pm$ S.E.M.) of 0.6 $\pm$ 0.2 for CaMKII, 0.8 $\pm$ 0.1 for PKA, 0.2 $\pm$ 0.02 for PKC and 1.34 $\pm$ 0.3 for CKII. Two-dimensional phosphoamino acid analysis of these phosphoprotein bands indicated that in all cases phosphorylation was primarily on serine residues, with minor phosphorylation on threonine by PKC (Figure 2B).

Time and dose dependence of Rad phosphorylation

Figure 3 explores the time course and dose dependence of Rad phosphorylation. In each case, phosphorylation was detectable within 5 min and reached a peak by 45 min at 30 °C for PKC (Figure 3A) and CKII (Figure 3E), and within 30 min for PKA (Figure 3C). Phosphorylation was also dependent on the quantity of kinase used in the reaction (Figures 3B, 3D and 3F). No significant phosphorylation of GST alone was observed under these conditions for each kinase tested.

Phosphorylation occurs at the C-terminus of Rad

In an attempt to localize the sites of Rad phosphorylation *in vitro*, we utilized two deletion constructs: C249, a construct lacking the C-terminal 60 residues of Rad, including four potential phosphorylation sites, and C297, a construct lacking

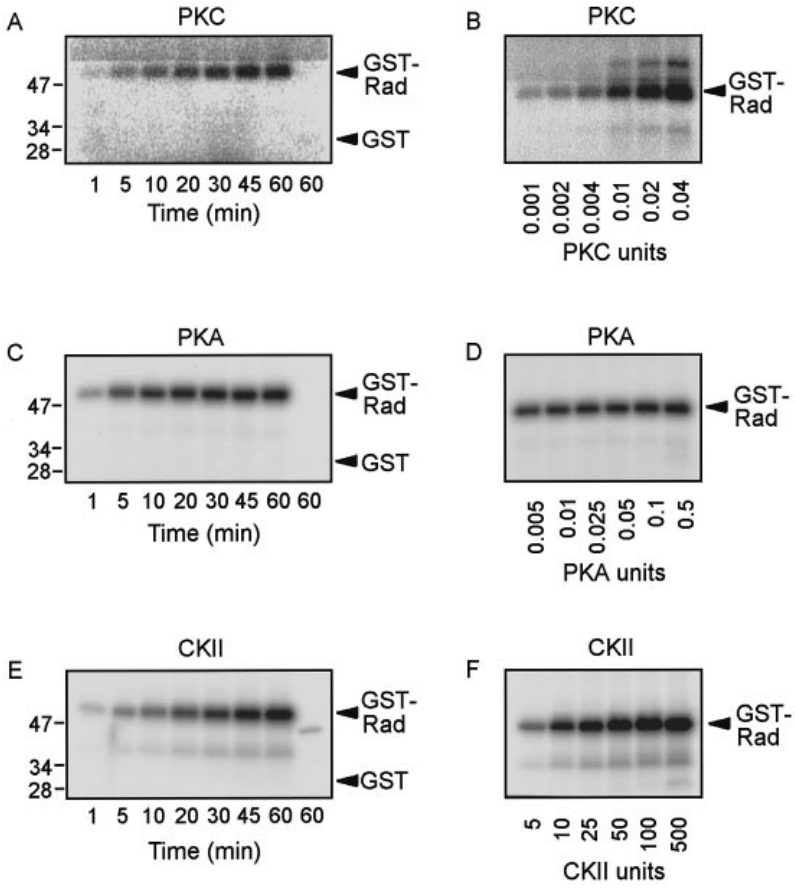

Figure 3 Time and dose dependence of Rad phosphorylation

GST–Rad (5 μg) or GST (5 μg) (the last lane in each of **A**, **C** and **E**) was incubated for various times at 30 °C in a kinase reaction *in vitro* containing [γ-^{32}P]ATP and 0.02 unit of PKC (**A**), 0.05 unit of PKA (**C**) or 50 units of CKII (**E**), as described in the Materials and methods section. For dose determinations 5 μg of GST–Rad was incubated for 45 min at 30 °C in a kinase reaction *in vitro* containing [γ-^{32}P]ATP and the indicated concentrations of PKC (**B**), PKA (**D**) or CKII (**F**), before analysis by SDS/PAGE and autoradiography.

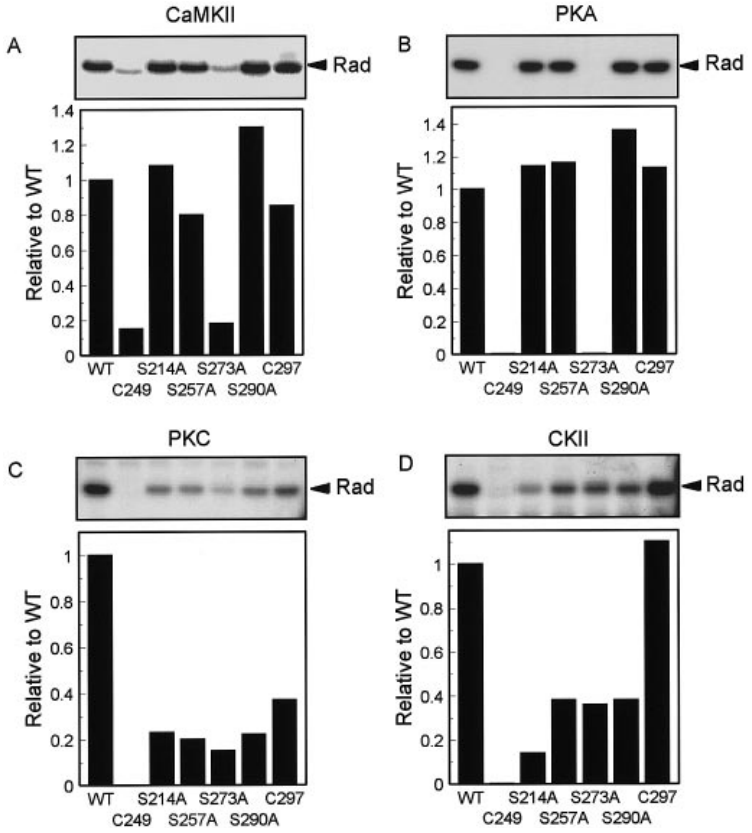

Figure 4 Phosphorylation occurs at the C-terminus of Rad

WT or mutant GST–Rad (5 μg) was incubated in a kinase reaction *in vitro* with CaMKII (100 units, **A**), PKA (0.05 unit, **B**), PKC (0.02 unit, **C**) or CKII (50 units, **D**) as described in the Materials and methods section. Results were quantified with a Molecular Dynamics PhosphorImager and are expressed relative to WT Rad. Samples C249 and C297 contained C-terminal deletions of Rad terminating at residues 249 and 297 respectively. Serine mutants S214A, S257A, S273A and S290A contained serine-to-alanine substitutions at the indicated positions.

the final 11 residues of Rad, including one potential phosphorylation site. In addition, constructs S214A, S257A, S273A and S290A were made, containing point mutations at several consensus sites of phosphorylation that converted Ser^{214}, Ser^{257}, Ser^{273} and Ser^{290} to alanine residues. Each of these constructs was purified as a GST fusion protein and used *in vitro* in kinase reactions as substrates for CaMKII, PKA, PKC or CKII.

Phosphorylation of Rad by CaMKII and PKA was diminished by more than 85% after removal of the C-terminal 60 residues of Rad (C249) or by conversion of a single serine residue at position 273 to alanine (Figures 4A and 4B). Mutation at other potential sites of phosphorylation was without effect (Figures 4A and 4B). Ser^{273} conforms to the consensus motifs of phosphorylation for both CaMKII [RXXS/T] and PKA [RXS/T] or [RR/KXS/T] [17]. Analysis of Rad mutants as substrates for PKC revealed that removal of the C-terminus of Rad also abolished phosphorylation. In contrast with the results for CaMKII and PKA, however, no single mutation reproduced the result, but mutation of Ser^{214}, Ser^{257}, Ser^{273} or Ser^{290} or the deletion of residues 298–308 all resulted in some diminished efficiency of phosphorylation (Figure 4C). This is in agreement with the fact that Ser^{214}, Ser^{257}, Ser^{273} and Ser^{290} each conform to the consensus sites of PKC phosphorylation [S/TXK/R], [K/RXXS/T] or [K/RXS/T] [17], as does Ser^{299}, which is deleted in the C297 construct. In contrast, whereas only Ser^{214} is a consensus site of phosphorylation by CKII [S/TXXE] [17], removal of the C-terminus in construct C249 and mutation of residues 215, 257, 273 and 290 each diminished Rad's ability to serve as a substrate, although deletion of residues 298–308 did not (Figure 4D). These findings indicate that Rad is phosphorylated by CaMKII and PKA primarily on Ser^{273}. PKC phosphorylates Ser^{214}, Ser^{257}, Ser^{273}, Ser^{290} and most probably Ser^{299}, whereas CKII seems to phosphorylate all of these serine residues except Ser^{299}. Thus Rad serves as a substrate for several kinases with unique patterns of phosphorylation.

Phosphorylation does not affect GTP binding or hydrolysis by Rad

To determine whether phosphorylation affected the ability of Rad to act as a GTPase, recombinant GST–Rad was incubated in kinase reactions *in vitro* in the presence or absence of CaMKII, PKA, PKC or CKII before the assessment of the binding of GTP to Rad. In comparison with unphosphorylated Rad, incubation of Rad with CaMKII, PKC or CKII under phosphorylation conditions had no significant effect on Rad's ability to bind [^{3}H]GTP in a time-dependent manner (results not shown). In contrast, we consistently observed a 50–70% decrease in the ability of Rad to bind GTP after incubation with PKA, which did not occur in the presence of buffer only (Figure 5A). Surprisingly, when the S273A mutant of Rad, in which the serine residue phosphorylated by PKA had been converted to alanine, was utilized in a similar assay, incubation with PKA also diminished GTP binding by approx. 50% (Figure 5A), although no phosphorylation of Rad could be detected (Figure 4B). In addition, inclusion of the heat-stable inhibitor of PKA catalytic activity, PKI [18], did not prevent the decrease in GTP binding

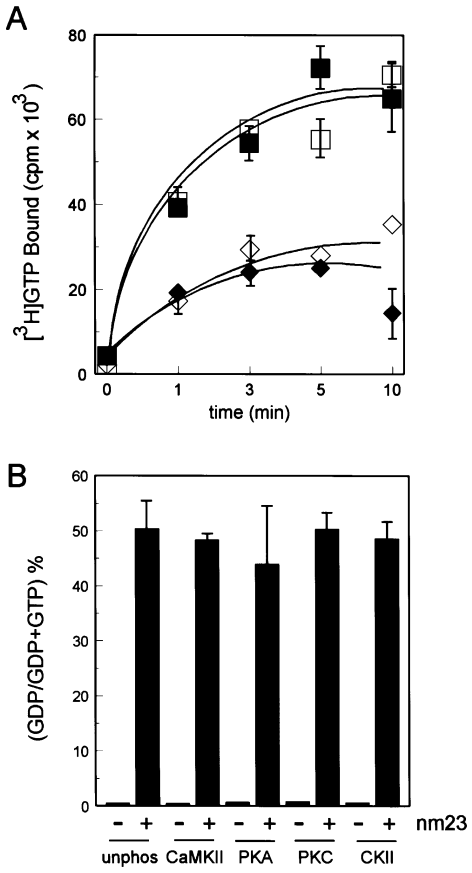

Figure 5 Phosphorylation does not affect Rad GTP binding or hydrolysis

(**A**) GST–Rad (■,◆) or the GST–Rad S273A mutant (□,◇) were incubated (20 pmol per reaction) in the presence of 100 μM ATP with (◆,◇) or without (■,□) 0.05 unit of PKA (1.3 pmol per reaction) for 45 min at 30 °C, as described in the Materials and methods section. Binding of [^{3}H]GTP (3 μCi per sample) was performed at room temperature. At various intervals duplicate aliquots were withdrawn, filtered through nitrocellulose and washed. The radioactivity bound to Rad was determined by scintillation counting. (**B**) For GTP hydrolysis 1 μg of GST–Rad bound to GSH–Sepharose beads was incubated for 45 min at 30 °C in a kinase reaction *in vitro* with 100 μM ATP and CaMKII, PKA, PKC or CKII. Reactions were washed three times in ice-cold loading buffer followed by incubation with 3 μCi of [γ^{32}P]GTP for 5 min at room temperature. GST–nm23 (500 ng) was added and hydrolysis was performed for 5 min at room temperature. After washing, bound nucleotides were eluted and equal radioactive counts were analysed by chromatography on poly(ethyleneimine)–cellulose TLC plates and PhosphorImager quantification. Results are expressed as the percentage conversion of GTP to GDP.

to Rad in the presence of PKA (results not shown). These findings suggest that the effect of PKA on GTP binding to Rad is not due to phosphorylation of Ser^{273} but is due to some other effect, perhaps a conformational change, brought about by the interaction of PKA with Rad, although in this experiment Rad was present in 15-fold molar excess.

For GTP hydrolysis assays, Rad was phosphorylated by CaMKII, PKA, PKC or CKII before incubation in the presence or absence of nm23, which serves to promote GTP hydrolysis by Rad (J. Zhu, J. D. Kantor, C. J. Rhodes, B. R. Zetter, J. S. Moyers and C. R. Kahn, unpublished work). In each case phosphorylation did not alter the intrinsic or nm23-induced GTPase activity of Rad in comparison with the activity observed with unphosphorylated Rad (Figure 5B).

Phosphorylation by PKC and CKII inhibits Rad binding to CaM

As noted above, several of the phosphorylation sites on Rad reside at the C-terminus of Rad, and this same domain is also

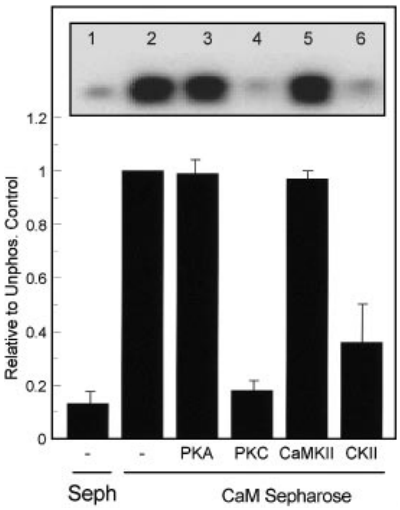

Figure 6 Phosphorylation by PKC and CKII inhibits binding of Rad to CaM

GST–Rad (500 ng) was incubated in a kinase assay *in vitro* for 45 min at 30 °C with 100 μM ATP and CaMKII, PKC, PKA or CKII. Ice-cold buffer (500 μl) was added, followed by incubation with CaM–Sepharose for 1 h at 4 °C. After washing, proteins bound to CaM–Sepharose were analysed by Western immunoblotting with anti-Rad antibody. Parallel kinase reactions performed in the presence of [γ-^{32}P]ATP revealed that Rad was phosphorylated with similar efficiencies to those in Figure 1 (results not shown). Results for three independent experiments were quantified by PhosphorImager analysis and are expressed as the amount of Rad bound to CaM–Sepharose relative to unphosphorylated Rad.

involved in CaM binding. To determine whether the phosphorylation of Rad affected the ability of Rad to bind CaM, GST–Rad (500 ng) was incubated in a kinase reaction *in vitro* with CaMKII, PKA, PKC or CKII and the ability of Rad to bind CaM-Sepharose was assessed. Western immunoblotting of proteins bound to CaM–Sepharose revealed that unphosphorylated Rad specifically bound CaM–Sepharose, but not control Sepharose beads (Figure 6, compare lane 2 with lane 1). Phosphorylation by CaMKII and PKA had no effect on Rad's ability to interact with CaM in comparison with unphosphorylated Rad (Figure 6, lanes 3 and 5). In contrast, phosphorylation by PKC and CKII abolished the binding of Rad to CaM (Figure 6, lanes 4 and 6). Quantification of three independent experiments revealed that the binding of Rad to CaM was decreased by more than 80% after PKC phosphorylation and by more than 60% after CKII phosphorylation, relative to unphosphorylated controls (Figure 6, lower panel). Incubation of Rad in each buffer in the absence of kinase had no effect on binding to CaM (results not shown). These results suggest that the phosphorylation of one or more of the C-terminal residues of Rad, most probably on Ser^{214}, Ser^{257} or Ser^{290}, which are each phosphorylated by PKC and CKII, disrupts the interaction of CaM with the C-terminus of Rad.

DISCUSSION

Rad is a novel Ras-related GTPase that is highly expressed in skeletal and cardiac muscle and has been suggested to have possible roles in insulin resistance to glucose uptake, cytoskeletal arrangement and Ca^{2+} signalling [1,4–6,19]. We have shown previously that Rad binds CaM in a Ca^{2+}-dependent manner via the C-terminus of Rad, which is extended relative to other Ras family members and lacks a typical prenylation motif [6]. This region of Rad, consistent with other CaM-binding domains, is predicted to form an amphipathic α-helical structure with hydro-

phobic and positively charged residues residing on opposite sides of the helix [6]. We have previously found that, in addition to binding CaM, Rad interacts with CaMKII (the downstream effector of CaM) and serves as a substrate for this kinase *in vitro* [6]. Indeed, several consensus sites for the phosphorylation of Rad reside in the extended C-terminus, proximal to the CaM-binding domain. In the present study we have characterized the phosphorylation of Rad by CaMKII, PKA, PKC and CKII, determined the sites of phosphorylation by mutagenesis and determined the effect of phosphorylation in the GTPase activity and CaM-binding properties of Rad.

We find that Rad was efficiently phosphorylated by CaMKII, PKA and CKII and, to a smaller extent, PKC. As expected, phosphorylation by all four of the kinases occurs almost exclusively on serine residues. Deletion and point mutation analysis revealed that Ser^{273} is the primary site of phosphorylation by CaMKII and PKA, whereas PKC and CKII phosphorylate several residues at the C-terminus of Rad, including Ser^{214}, Ser^{257}, Ser^{273}, Ser^{290} and Ser^{299}. Although it is unclear why single point mutations produce a major decrease in phosphorylation of Rad by PKC, it is unlikely that these mutations disrupt the overall structure of Rad because, with the exception of S273A, these same mutants are efficiently phosphorylated by CaMKII and PKA.

We assessed whether phosphorylation has a direct effect on the ability of Rad to bind or hydrolyse GTP in the presence of nm23, which we have recently found to promote GTP hydrolysis by Rad, i.e. to serve as a Rad-GTPase activating protein (J. Zhu, J. D. Kantor, C. J. Rhodes, B. R. Zetter, J. S. Moyers and C. R. Kahn, unpublished work). Although we observed no effects on intrinsic or nm23-induced GTP hydrolysis by Rad, we found that preincubating Rad with PKA significantly decreased the ability of Rad to bind GTP. Interestingly, however, this effect does not seem to be due to phosphorylation of Rad because the same decrease in GTP binding occurred with the S273A construct containing a mutation at the single site of phosphorylation by PKA. Thus it seems that PKA affects the guanine nucleotide binding of Rad by interacting with the protein in a manner not dependent on the catalytic activity of PKA. Perhaps PKA interacts directly with Rad, inducing a conformational change that alters GTP binding. Further studies are needed to clarify the mechanism of this effect.

A more striking effect of phosphorylation of Rad by PKC and CKII is a marked decrease in the binding of this GTPase to CaM. This is similar to previous reports that the phosphorylation by PKC of neuromodulin and myristoylated alanine-rich C-kinase substrate (MARCKS) proteins, near the site of CaM binding, blocks their interaction with CaM [20,21]. Comparison of the identified sites of phosphorylation in Rad by each kinase suggests that phosphorylated Ser^{214}, Ser^{257} and Ser^{290} are possible candidates to regulate CaM binding negatively. Ser^{214} and Ser^{257} lie proximal to the identified site of CaM binding (residues 278–297), whereas Ser^{290} is within the CaM-binding domain of Rad. Thus it seems likely that introduction of negative charge at Ser^{290} disrupts the positively charged environment and thus the interaction with CaM. Because inhibition of CaM binding is observed even with the relatively low stoichiometry of phosphorylation of Rad by PKC, it is possible that more than one molecule of Rad is required to form a full CaM-binding complex.

The ability of Rad to serve as a phosphoprotein places it within the small group of low-molecular-mass GTPases that are phosphorylated, including K-ras, Rab4, smg p21, Rap1B and Gem [8–14]. Whereas the phosphorylation-dephosphorylation of Rab4 regulates translocation, the roles of phosphorylation of the other proteins are unclear. In Rad, phosphorylation disrupts CaM binding, suggesting that phosphorylation might regulate Ca^{2+}-mediated signalling through Rad. We have previously shown that the removal of the C-terminal CaM-binding site of Rad results in the disruption of Rad from cytoskeletal and membrane fractions of C2C12 cells [6]. Coupled with the present finding, this suggests that the C-terminal domain mediates the intracellular localization of Rad either directly, or indirectly via CaM binding. In a somewhat similar manner, Rab4 is phosphorylated at Ser^{196} *in vivo* in mitotic cells and by $p34^{cdc2}$ kinase *in vitro* [12], and mutation of this residue prevents the accumulation of Rab4 into the cytosol in mitotic Chinese hamster ovary cells. Thus perhaps the phosphorylation of the C-terminus of Rad, like that of Rab4, regulates localization of the protein.

In summary, the Rad-like proteins are a unique category of GTP-binding molecules with unique and complex regulation. Serine phosphorylation of Rad by multiple kinases might have a role in the regulation of a number of properties of this molecule, including its CaM binding, localization and GTP binding.

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