Members of the Rad family of GTPases (including Rad, Gem, and Kir) possess several unique features of unknown function in comparison to other Ras-like proteins, with major N-terminal and C-terminal extensions, a lack of typical prenylation motifs, and several non-conservative changes in the sequence of the GTP binding domain. Here we show that Rad and Gem bind to calmodulin (CaM)–Sepharose in vitro in a calcium-dependent manner and that Rad can be co-immunoprecipitated with CaM in C2C12 cells. The interaction is influenced by the guanine nucleotide binding state of Rad with the GDP-bound form exhibiting 5-fold better binding to CaM than the GTP-bound protein. In addition, the dominant negative mutant of Rad (S105N) which binds GDP, but not GTP, exhibits enhanced binding to CaM in vivo when expressed in C2C12 cells. Peptide competition studies and expression of deletion mutants of Rad localize the binding site for CaM to residues 278–297 at the C terminus of Rad. This domain contains a motif characteristic of a calmodulin-binding region, consisting of numerous basic and hydrophobic residues. In addition, we have identified a second potential regulatory domain in the extended N terminus of Rad which, when removed, decreases Rad protein expression but increases the binding of Rad to CaM. The ability of Rad mutants to bind CaM correlates with their localization in cytoskeletal fractions of C2C12 cells. Immunoprecipitates of calmodulin-dependent protein kinase II, the cellular effector of Ca2+-calmodulin, also contain Rad, and in vitro both Rad and Gem can serve as substrates for this kinase. Thus, the Rad family of GTP-binding proteins possess unique characteristics of binding CaM and calmodulin-dependent protein kinase II, suggesting a role for Rad-like GTPases in calcium activation of serine/threonine kinase cascades.

Rad is the prototypic member of a new class of Ras-like GTP-binding proteins that includes Gem and Kir (1–3). In humans, Rad is most highly expressed in the heart, lung, and skeletal muscle and expression is increased in the skeletal muscle of some type II diabetic humans (1). Rad exhibits a unique magnesium dependence for guanine nucleotide binding and is regulated by a Rad-specific GTPase-activating protein (GAP)1 (4). In cultured muscle and fat cells, Rad overexpression attenuates insulin-stimulated glucose uptake without altering expression or insulin-stimulated translocation of the Glu4 glucose transporter (5). By expression library screening, Rad has been shown to interact with skeletal muscle β-tropomyosin, suggesting that Rad may participate in regulation of the cytoskeleton (6). The Gem gene product is expressed in the G1 phase in mitogen-activated T lymphocytes and sharesapproximately 60% amino acid identity with Rad (2), whereas kir was isolated from a pre-B-cell library and is overexpressed in cells expressing BCR/ABL or v-abl (3). Murine kir and gem are 98.4% identical in nucleotide sequence and encode the same or very highly related proteins, referred to here as Kir/Gem (3).

When expressed in Saccharomyces cerevisiae, kir induces invasive pseudohyphal growth and may function upstream of the STE20 kinase (7).

rad and kir/gem encode GTP-binding proteins with several structural features that are distinct from other GTPases (1–3). The N terminus of Rad is extended by 88 amino acids, and Kir/Gem is extended by 72 amino acids in comparison to Ras, and the C terminus of each is extended by 31 amino acids. Although Rad and Kir/Gem share 100% identity in the final 11 amino acids, they lack a CAAAX-like prenylation site present in other Ras-like molecules (8, 9). Rad and Kir/Gem differ from each other and from other Ras-like proteins in the putative effector (G2) domain, suggesting that they interact with distinct GAPs or effector molecules. They also contain residues in the G3 consensus sequence for guanine nucleotide binding which are divergent from Ras (1–3).

Members of the Ras family of GTP-binding proteins participate in a number of cellular functions including proliferation (10), vesicular transport (11, 12), and cytoskeletal arrangement (13, 14). Rac and cdc42 have been shown to interact with phosphatidylinositol 3-kinase and to participate in signaling leading to activation of the Jun kinases (15, 16). While the exact function of Rad and Kir/Gem is unknown, it has recently been reported in abstract that peptides based on the C terminus of Rad and Kir/Gem can bind to calmodulin (CaM) in vitro (17). In this study, we show that the full-length Rad protein binds CaM in vitro and in vivo in a Ca2+-dependent manner and that the C-terminal residues 278–297 of human Rad are critical for this interaction. The binding of Rad and CaM is influenced by the guanine nucleotide bound state of Rad. We also demonstrate that Rad is present in complex with the cellular target of CaM, calmodulin-dependent protein kinase II (CaMKII), which can phosphorylate both Rad and Gem in vitro.

1 The abbreviations used are: GAP, GTPase-activating protein; CaM, calmodulin; CaMKII, calmodulin-dependent protein kinase II; GTP-S, guanosine 5′-O-(2-thiodiphosphate); DTT, dithiothreitol; GST, glutathione S-transferase; MBP, myelin basic protein; Puro, puromycin; WT, wild type.

‡ To whom correspondence should be addressed: Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. Tel.: 617-732-2635; Fax: 617-732-2593.
These findings suggest that the Rad family of Ras-like proteins may participate in Ca$^{2+}$-triggered signaling events involving CaM and the CaMKII serine/threonine kinase cascade.

**EXPERIMENTAL PROCEDURES**

**Construction of Rad Mutants and Transduction of Cell Lines—**

C2C12 murine myotubes transfected with puromycin resistance vector only (Puro), or expressing full-length human wild-type (WT) rad cDNA, the potential dominant negative mutant, S105N, or the putative activat

**RESULTS**

**Rad Interacts with CaM and CaMKII**

To determine whether Rad binds CaM, lysates from C2C12 myoblasts over-expressing Rad were incubated with calmodulin-Sepharose in the presence of 1 mM CaCl$_2$ or 2 mM EGTA as described under "Experimental Procedures." In the presence of Ca$^{2+}$, but not EGTA, Rad bound CaM-Sepharose, but not the control GST-Sepharose (Fig. 1A). Immunoprecipitation of the supernatant from the CaM-Sepharose experiment with anti-Rad antibody revealed that nearly all of the Rad protein was depleted by prior binding to CaM-Sepharose in the presence of Ca$^{2+}$, while the immunoprecipitation of Rad from control samples was unaffected by CaCl$_2$ or EGTA treatment (Fig. 1B). Similar experiments were performed with purified Gem incubated with CaM-Sepharose. Like Rad, Gem associated with CaM in the presence of 1 mM CaCl$_2$ (Fig. 1C, lane 2), and this could be disrupted by treatment with 2 mM EGTA (Fig. 1C, lane 3). No detectable protein was associated with the control Sepharose 4B beads in the presence of 1 mM CaCl$_2$ (Fig. 1C, lane 1). Western immunoblotting revealed that equal quantities of Gem were detected in the starting material in the presence of CaCl$_2$, or EGTA (Fig. 1D).
cells with 5 μM ionomycin prior to lysis did not appear to affect the amount of Rad bound to CaM; however, it is likely that endogenous stores of Ca^{2+} released upon lysis might have already induced the binding of these proteins in the absence of added Ca^{2+}. Consistent with this, lysis of cells in the presence of 2 mM EGTA disrupts Rad and CaM interaction (not shown). In addition, when CaM immune complexes were washed with 2 mM EGTA, Rad was completely dissociated (Fig. 2A, lane 3), whereas the quantity of Rad in anti-Rad immune complexes was unaffected by this treatment (Fig. 2A, lane 6 versus lanes 4 and 5), suggesting Ca^{2+} dependence of the Rad-CaM interaction in vivo.

Immunoprecipitation with anti-Rad antibody followed by Western immunoblotting for CaM revealed the presence of CaM in anti-Rad immune complexes (Fig. 2B, lanes 4 and 5 and the dissociation of this complex by EGTA treatment (Fig. 2B, lane 6). In the presence of EGTA, CaM exhibited the expected altered migration pattern (Fig. 2B, lane 3) (19). Thus, Rad and CaM co-precipitate in immune complexes in a Ca^{2+}-dependent manner.

CaM Binds the C Terminus of Rad—CaM-binding sites are rich in hydrophobic and basic residues with the potential to form a basic amphipathic α-helix, often with charged and hydrophobic residues residing on opposite sides of the helix (20). When modeled as a helical wheel, Rad C-terminal residues 274–291 form an almost ideal CaM-binding domain with a structure composed of opposing surfaces rich in charged residues or long chain hydrophobic amino acids (Fig. 3).

To determine whether Rad binds CaM via C-terminal residues, we assessed the ability of a peptide corresponding to the final 30 amino acids of Rad to compete for Rad binding to CaM-Sepharose. As shown in Fig. 4A, increasing concentrations of a peptide corresponding to residues 278–308 of human Rad competitively inhibited the interaction of Rad and CaM, whereas the same concentrations of a peptide corresponding to the effector region of Rad (residues 115–127) did not. Quantitation of these results revealed an IC_{50} of 5 μM for incubation in this assay when the concentration of CaM was approximately 3 μM (Fig. 4B), suggesting an approximate 1:1 stoichiometry of Rad-CaM interaction.

To further analyze the CaM-binding region of Rad, several deletion mutants of Rad were constructed and expressed in C2C12 cells. The Rad C297 construct terminates at residue 297, deleting the final 11 amino acids of Rad which share 100% identity with Kir/Gem (1–3). The Rad C278 construct deletes...

FIG. 1. Rad and Gem bind CaM in a Ca^{2+}-dependent manner. 500 μg of lysates from C2C12 cells expressing WT Rad were incubated with CaM-Sepharose or with the control GST-Sepharose in the presence of 2 mM EGTA (lanes 1 and 3) or 1 mM CaCl_{2} (lanes 2 and 4), as described under “Experimental Procedures.” The precipitates were subjected to Western immunoblotting using anti-Rad antibody (A), and the supernatants from these incubations were subjected to immunoprecipitation using anti-Rad antibody followed by Western immunoblotting for Rad (B). C, recombinant Gem (0.5 μg), purified by thrombin cleavage from GST-Gem fusion protein, was incubated with Sepharose 4B beads (lane 1) or CaM-Sepharose in the presence of 1 mM CaCl_{2} (lane 2) or 2 mM EGTA (lane 3) followed by Western immunoblotting using anti-Gem antibody. The positions of Gem and an apparent degradation product are indicated. Portions (1/100) of the starting material for C were subjected to Western immunoblotting using anti-Gem antibody (D).

FIG. 2. Co-immunoprecipitation of Rad and CaM. Following overnight serum starvation, C2C12 cells expressing WT Rad were treated with or without 5 μM ionomycin for 5 min. Lysates (1 mg) were subjected to immunoprecipitation (IP) using anti-CaM or anti-Rad antibodies followed by Western immunoblotting with anti-Rad antibody (A) or anti-CaM antibody (B) as described under “Experimental Procedures.” B, 50 ng of purified CaM and 100 μg of total cellular lysate are included.

FIG. 3. Potential CaM binding region of Rad. A helical wheel representation of Rad residues 275–293 reveals an amphipathic secondary structure. Long chain hydrophobic residues are circled. Positively charged residues are indicated as +.
Rad Interacts with CaM and CaMKII

500 μg of lysates from C2C12 cells expressing WT Rad were incubated with 10 μl of CaM-Sepharose or GST-Sepharose in a final volume of 500 μl (approximately 3 μl final CaM concentration) as described under "Experimental Procedures." Incubations were carried out in the presence of 1 mM CaCl2, 2 mM EGTA, or various concentrations of a Rad C-terminal peptide 278–308 or a Rad effector domain peptide in the presence of 1 mM CaCl2 (A). Precipitates were analyzed by Western immunoblotting for Rad. Results were quantitated using a Molecular Dynamics PhosphorImager (B). C, 1 mg of lysates from puromycin-resistant C2C12 control cells (Puro, lanes 1 and 2) or cells expressing WT (lanes 3 and 4) and mutant Rad were incubated with CaM-Sepharose in the presence of 1 mM CaCl2 or 2 mM EGTA. Bound proteins (C) and 10 μg of each lysate sample (D) were analyzed by Western immunoblotting for Rad. C and D, the numbering of C-terminal deletion mutants, C297 (lanes 5 and 6), C278 (lanes 7 and 8), and C249 (lanes 9 and 10), indicates the residue where the truncation mutant terminates. The position of the N-terminal deletion mutant N88 (lanes 11 and 12), which lacks Rad residues 1–88, is indicated. Endogenous Rad in puromycin (Puro) cells (C, lane 1 and D, lanes 1 and 2) and the N88 Rad mutant (D, lanes 11 and 12) are visible following long exposure (not shown). Results were quantitated using a Molecular Dynamics PhosphorImager and are expressed as the quantity of protein bound to CaM (corrected for differences in expression) relative to WT Rad (E).

Fig. 4. CaM binds the C terminus of Rad. 500 μg of lysates from C2C12 cells expressing WT Rad were incubated with 10 μl of CaM-Sepharose or GST-Sepharose in a final volume of 500 μl (approximately 3 μl final CaM concentration) as described under "Experimental Procedures." Incubations were carried out in the presence of 1 mM CaCl2, 2 mM EGTA, or various concentrations of a Rad C-terminal peptide 278–308 or a Rad effector domain peptide in the presence of 1 mM CaCl2 (A). Precipitates were analyzed by Western immunoblotting for Rad. Results were quantitated using a Molecular Dynamics PhosphorImager (B). C, 1 mg of lysates from puromycin-resistant C2C12 control cells (Puro, lanes 1 and 2) or cells expressing WT (lanes 3 and 4) and mutant Rad were incubated with CaM-Sepharose in the presence of 1 mM CaCl2 or 2 mM EGTA. Bound proteins (C) and 10 μg of each lysate sample (D) were analyzed by Western immunoblotting for Rad. C and D, the numbering of C-terminal deletion mutants, C297 (lanes 5 and 6), C278 (lanes 7 and 8), and C249 (lanes 9 and 10), indicates the residue where the truncation mutant terminates. The position of the N-terminal deletion mutant N88 (lanes 11 and 12), which lacks Rad residues 1–88, is indicated. Endogenous Rad in puromycin (Puro) cells (C, lane 1 and D, lanes 1 and 2) and the N88 Rad mutant (D, lanes 11 and 12) are visible following long exposure (not shown). Results were quantitated using a Molecular Dynamics PhosphorImager and are expressed as the quantity of protein bound to CaM (corrected for differences in expression) relative to WT Rad (E).

The final 30 amino acids, corresponding to the presumed CaM-binding domain, and the Rad C249 construct deletes the final 59 amino acids of Rad. As in previous experiments, incubation of lysates of C2C12 cells overexpressing WT Rad with CaM-Sepharose followed by Western blotting revealed Ca2+-dependent binding of Rad to the CaM-Sepharose (Fig. 4C, lane 3), which was disrupted by EGTA treatment (Fig. 4C, lane 4). Endogenous Rad in control cells (Fig. 4C, lanes 1 and 2) also bound in a Ca2+-dependent manner and was visible upon longer exposure of the blots (not shown). Deletion of the C-terminal residues 297–308 (C297 mutant) did not significantly affect the ability of Rad to bind CaM (Fig. 4C, lanes 5 and 6). In contrast, deletion of C-terminal residues 278–308 (C278 mutant) abolished Rad binding to CaM (Fig. 4C, lanes 7 and 8), as did a larger deletion of residues 249–308 (C249 mutant, Fig. 4C, lanes 9 and 10), which creates a molecule of identical length to Ras at the C terminus.

As noted above, in addition to the C-terminal extension, Rad also contains 88 additional amino acids on the N terminus as compared with Ras. To identify whether the N terminus of Rad contributes to the binding to CaM, a deletion was made that removes the unique N terminus of Rad. This mutant (N88) exhibited much lower levels of expression than the other mutants (Fig. 4D, lanes 11 and 12), but an appropriate length protein could still be detected by Western blotting of lysates following longer exposure (not shown). Upon incubation of cell lysates with CaM-Sepharose, Rad-88 exhibited a striking enhancement in Ca2+-dependent binding relative to WT Rad (Fig. 4C, lane 11). Quantitation of Rad protein bound to CaM relative to the levels of expression revealed that CaM binding was decreased by >90% with the Rad C278 and C249 C-terminal deletion mutants, whereas the Rad N88 N-terminal mutant exhibited an approximate 24-fold enhancement in CaM binding relative to the wild type protein (Fig. 4E). Thus, the residues of Rad critical for CaM binding are contained within the region encompassing the C-terminal residues 278–297, whereas the N terminus of Rad appears to encode a region that may affect antibody recognition, protein expression or stability, as well as a potential negative regulatory region for CaM binding.

GDP-Rad Preferentially Binds CaM—To determine whether the interaction of Rad and CaM is influenced by the guanine nucleotide-bound state of Rad, the binding of GTP-Rad to CaM-Sepharose was assessed following preloading of Rad with GDP or GTPγS. In vitro GDP-bound Rad preferentially bound CaM as compared with GTPγS-bound Rad with an approximate 8-fold increase in the quantity of Rad protein bound (Fig. 5A). Removal of Ca2+ by EGTA treatment disrupted binding of CaM, regardless of guanine nucleotide association. Similar results were obtained when Rad protein was cleaved from GST by thrombin treatment prior to the assay (not shown).

To further assess the influence of guanine nucleotide binding on Rad and CaM interaction in vivo, C2C12 cells were used that overexpress wild type Rad (WT), Rad with a mutation (S105N) that abolishes GTP-binding activity and favors GDP binding, and is thus a potential dominant-negative molecule, or Rad with a double mutation (P61V/Q109H, PVQH) that results in a molecule with high intrinsic GTP binding and GTPase activities in vitro (4). Lysates of these cells were assayed by Western immunoblotting for Rad binding to CaM-Sepharose (Fig. 5B) and for expression levels of the Rad proteins (Fig. 5C). The binding to CaM relative to the expression level of WT, S105N, and PVQH Rad was quantitated in Fig. 5D. Results from three independent experiments revealed that the Rad S105N mutant exhibited an approximate 5-fold enhanced CaM binding relative to the WT and PVQH proteins, despite lower relative expression of this mutant. The binding of all three proteins was disrupted by EGTA treatment (Fig. 5B). These results, together with those in which GDP incubation enhanced binding, suggest that the GDP-bound form of Rad preferentially associates with CaM.

CaM Does Not Affect Rad GTPase Activity—A possible role of CaM binding might be to alter the GTPase activity of Rad. To test this hypothesis Rad and Rad bound to CaM were subjected to a GTP hydrolysis assay in the presence or absence of a preparation of partially purified Rad-GAP (4). GTP hydrolysis by Rad was stimulated approximately 2.5-fold in the presence of partially purified Rad-GAP (Fig. 6A). Rad bound to CaM exhibited a slight elevation in Rad-GAP-stimulated GTP hydrolysis; however, this was not significantly different from controls. In addition, CaM had no effect on intrinsic Rad GTPase activity in the absence of Rad-GAP. Since CaM preferentially binds the GDP form of Rad, we speculated that CaM might affect the ability of Rad to bind GTP. However, binding studies performed in the presence or absence of 5-fold molar...
excess of CaM did not show an affect of CaM binding on the ability of Rad to bind either [3H]GTP (Fig. 6B) or [3H]GDP (Fig. 6C), suggesting that CaM binding may not regulate guanine nucleotide binding of Rad.

Rad Is Present in Complex with CaMKII—A major cellular effector of Ca\(^{2+}\) -CaM signaling is the serine/threonine kinase CaMKII (20, 21). To determine whether Rad exists in complex with CaMKII, lysates from control cells, cells expressing WT Rad, and cells expressing Rad mutants that exhibited altered binding to CaM (C278, N88, and S105N) were subjected to immunoprecipitation with anti-CaMKII antibody followed by Western immunoblotting to detect Rad (Fig. 7A). WT Rad overexpressed in C2C12 cells was detected in anti-CaMKII immunoprecipitates; endogenous Rad was detectable in these immunoprecipitates upon longer exposure (not shown). Comparison of blots of whole cell lysates indicated that approximately 2% of the total cellular Rad protein was present in anti-CaMKII immunoprecipitates. Quantitation of Rad protein bound to anti-CaMKII immune complexes relative to the levels of expression is shown in Fig. 7C. In contrast to what was observed with the CaM binding experiments, the C-terminal Rad mutant, C278, truncated at residue 278 exhibited similar binding to CaMKII immune complexes as compared with the WT and endogenous Rad (Fig. 7, A and C). The N-terminal truncation, N88 (Fig. 7A, lane 4) exhibited an approximate 4-fold enhancement in binding relative to WT Rad despite very low levels of expression of this mutant (Fig. 7B, lane 4). A longer exposure of this lane is shown, and the migration of the 88 mutant is

![Figure 5](http://www.jbc.org/)

**Fig. 5.** GDP-Rad preferentially binds CaM. GST-Rad (0.5 μg) was incubated with 0.5 mM GDP or 0.5 mM GTP\(\gamma\)S at room temperature for 30 min prior to the addition of 20 μl of CaM-Sepharose. Following incubation at 4 °C for 4 h, beads were washed and analyzed by Western immunoblotting for Rad (A). Lysates (500 μg) from C2C12 cells expressing WT Rad, a potential dominant negative Rad (S105N), or a mutant (PVQH) which exhibits elevated GTP binding and hydrolysis *in vitro* were incubated with CaM-Sepharose in the presence of 1 mM CaCl\(_2\) or 2 mM EGTA (B). 10 μg of the lysates was subjected to Western immunoblotting to detect the level of Rad expression (C). The results from three independent experiments were quantitated using a Molecular Dynamics PhosphorImager and are expressed as the % of total Rad protein ± S.E. (corrected for differences in expression) bound to CaM (D).

![Figure 6](http://www.jbc.org/)

**Fig. 6.** CaM does not affect Rad GTPase activity. 1 μg of purified GST-Rad was incubated with a 10-fold molar excess of purified CaM for 1 h at 4 °C prior to assaying for GTPase activity in the presence (+) or absence (−) of partially purified Rad-GAP as described under “Experimental Procedures.” Reaction products were separated by thin layer chromatography and quantitated using a PhosphorImager. Results are represented as %GDP hydrolyzed per total guanine nucleotide bound (GDP + GTP) ± S.E. relative to control samples for four independent experiments. 20 pmol of GST-Rad was incubated with [\(^{3}\)H]GTP (3 μCi/1.7 μM) or [\(^{3}\)H]GDP (3 μCi/1.7 μM) in the presence (squares) or absence (circles) of 100 pmol of purified CaM for the indicated times at room temperature, as described under “Experimental Procedures.” Reaction products were applied to nitrocellulose filters, washed, and the radioactivity remaining on the filters determined by scintillation counting. Results are expressed as counts bound ± S.E. for [\(^{3}\)H]GTP binding (A) and [\(^{3}\)H]GDP binding (B) and are representative of three or two independent experiments, respectively, each performed in duplicate.
Thus, Rad is present in complex with CaMKII under conditions that did not disrupt Rad-CaMKII binding (Fig. 7, lane 5). In addition, the N-terminal deletion mutant, N88 (lane 4), or the S105N dominant-negative mutant (lane 5) were subjected to immunoprecipitation with anti-CaMKII antibody followed by Western blotting for Rad, as described under “Experimental Procedures” (A). 30 μg of each lysate sample was subjected to Western blotting for Rad expression (B, lanes 1–5). Lane 6 is a long exposure of lane 4, showing the migration of the N-terminal mutant, N88. Results were quantitated using a Molecular Dynamics PhosphorImager, as described in the legend to Fig. 4, and are expressed as the quantity of protein bound to CaMKII (corrected for differences in expression) relative to the WT Rad protein (C). D, 500 μg of lysate from cells expressing WT Rad was subjected to immunoprecipitation with anti-CaMKII antibody followed by Western immunoblotting for Rad. Immunoprecipitations were carried out in lysis buffer (lane 1) or in the presence of 1 mM CaCl2 (lane 2), 2 mM EGTA (lane 3), or the Rad C-terminal peptide 278–308 at 10 (lane 4) or 50 μM (lane 5).

indicated in Fig. 7B, lane 6. Similar to the results for CaM binding, the S105N mutant (Fig. 7A, lane 5) exhibited an approximate 8-fold enhanced binding to CaMKII, suggesting that this interaction may also be favored by the GDP-bound form of Rad. Since the Rad truncated at residue 278 (C278) retained the ability to bind CaMKII but was unable to bind CaM, further experiments were performed to confirm that Rad binding to CaMKII is independent of its interaction with CaM. Thus, lysates from cells expressing WT Rad were subjected to immunoprecipitation with anti-CaMKII antibodies followed by EGTA washing of the immune complexes, a treatment which results in dissociation of Rad and CaM. As shown in Fig. 7D, the presence of 1 mM CaCl2 or 2 mM EGTA did not affect the Rad-CaMKII complexes (compare lanes 2 and 3 versus lane 1). Likewise, addition of a peptide corresponding to residues 278–308 of Rad at concentrations that disrupt Rad-CaM interaction did not disrupt Rad-CaMKII binding (Fig. 7, lanes 4 and 5). Thus, Rad is present in complex with CaMKII under conditions in which Rad and CaM do not appear to interact.

Rad and Gem Are In Vitro Substrates for CaMKII—Rad and Kir/Gem possess several potential sites for CaMKII phosphorylation based on the presence of the consensus sequence RX(3)S/T (21). To determine whether these GTP-binding proteins can, in fact, serve as substrates for CaMKII, purified recombinant Rad and Gem were incubated in a kinase reaction containing CaMKII, CaM, and CaCl2, as described under “Experimental Procedures.” Both Rad and Gem were phosphorylated by CaMKII as was the control substrate, MBP (Fig. 8A). Thrombin, which was present in the protein preparations as a result of protein purification, was not phosphorylated by CaMKII. For a time course determination of Rad phosphorylation, GST-Rad or GST were incubated in a kinase reaction with 100 units of a truncated version of CaMKII at 30 °C for the indicated times prior to SDS-PAGE and autoradiography. C, 0.4 μg of GST-Rad was incubated with 0–500 units of truncated CaMKII at 30 °C for 45 min. The positions of GST-Rad, GST, and CaMKII in B and C are indicated.

Correlation of CaM Binding with Cellular Localization—Rad lacks the prenylation motifs found in most Ras-like molecules and is located in both the cytoplasm and membrane of the cell (5). A portion of Rad, however, is associated with the cytoskeleton and membrane skeleton components by as yet undeter-
Rad Interacts with CaM and CaMKII

DISCUSSION

Rad and Kir/Gem are members of a novel class of Ras-related GTP-binding proteins that contain unique and extended N and C termini as compared with other Ras-like proteins (1–3). In this study we have shown that these extended domains are involved in binding of CaM. Thus, Rad and Gem bind CaM-Sepharose in a Ca2+-dependent manner, and in cells, Rad co-immunoprecipitates with CaM in a manner that is disrupted by EGTA treatment. Therefore, the Rad family of proteins joins the growing list of CaM-binding proteins, including CaMII, myosin light chain kinase, phosphofructokinase, plasma membrane Ca2+-ATPase, neuromodulin, and, more recently, IQ-GAP1 and Ras-guanine nucleotide releasing factor (22–26).

Berchtold and Fischer (17) have shown that a peptide corresponding to the final C-terminal 30 amino acids of Kir/Gem binds to CaM in vitro, and in the current study we find that a synthetic peptide based on the final 30 amino acids of Rad (residues 278–308) competes for Rad binding to CaM-Sepharose. Further evidence that the CaM-binding domain of Rad is indeed located in the C-terminal extended region involved deletion mutants. Thus, while deletion of residues 297–308 from full-length Rad did not disrupt CaM binding, deletion of residues 278–308 abolished CaM binding completely. Based on these mutants, the specific residues critical for the CaM interaction lie in the 19-amino acid region encompassing residues 278–297 of human Rad. Modeling of this region as a helical wheel confirms the distribution of charged and hydrophobic residues typical of CaM-binding protein. It is likely that the corresponding region of Gem, which shares 79% homology to Rad and is also rich in charged and hydrophobic residues, mediates its binding to CaM. We have shown previously that in C2C12 cells, GDP-bound Rad binds skeletal muscle β-tropomyosin following Ca2+-ionophore treatment (6). Although the region of Rad that mediates this interaction has not been determined, addition of a 5-fold molar excess of purified tropomyosin did not affect the interaction of Rad and CaM, suggesting that the binding regions reside in different locations in Rad (not shown).

The Rad-CaM interaction appears to be dependent on the guanine nucleotide-bound state of Rad since GDP-loaded purified Rad and the Rad dominant negative mutant (S105N) expressed in cells exhibit increased binding to CaM in comparison to GTP-γS-loaded Rad and WT Rad in cells. We speculated that CaM may thus serve to sequester Rad in its inactive GDP-bound form, serving as a “switching off” mechanism; however, we failed to show an effect of CaM binding on the guanine nucleotide binding state of Rad, suggesting that this may not be the case. Alternatively, in the presence of Ca2+, Rad may serve to sequester CaM. Additionally, we have previously shown that treatment of C2C12 cells with Ca2+-ionophore, A23187, results in a rapid degradation of Rad protein (6). It is possible that Ca2+-CaM serves a role in the switching off of Rad by facilitating the degradation of Rad by Ca2+-activated proteases. CaM does not catalyze the inactivation of Rad by GTP hydrolysis, since CaM alone does not significantly affect Rad intrinsic GTPase activity nor Rad-GAP-stimulated GTP hydrolysis.

In addition to binding CaM, Rad exists in complex with CaMII, the serine/threonine kinase which is a cellular target of CaM. Deletion of the CaM-binding domain of Rad, treatment of immune complexes with EGTA, and competition studies with the CaM-binding domain peptide indicate that Rad interacts with CaMII independent of its association with Ca2+. CaM and that different domains of Rad are involved in these interactions. In addition, Rad and Gem serve as in vitro substrates for CaMII. Although the significance of this phosphorylation is not yet known, it is possible that CaMII modulates the function of Rad or its binding to CaM in a feedback mechanism. Two consensus sites for CaMII phosphorylation (serines 273 and 299) reside near the region of CaM binding (1) and could potentially modulate the Rad-CaM interaction by introducing a negative charge in the binding region.

It has been noted that several CaM-binding proteins, including CaMII and myosin light chain kinase, contain a “CaM-

P. J. Bilan, J. S. Moyers, and C. R. Kahn, manuscript submitted for publication.
like binding site" within the sequence of the molecule (i.e., rich in hydrophobic/anionic residues) which is proposed to act as an internal inhibitor of CaM binding by interacting with the hydrophobic/cationic CaM-binding site within the protein (22). Our mutagenesis studies suggest that Rad may have such a region in that deletion of the N-terminal 88 amino acids of Rad results in a molecule that exhibits enhanced binding to CaM-Sepharose. The region of Rad spanning residues 68–88 contains a number of hydrophobic and negatively charged residues, making it a potential auto-inhibitory domain (22). Thus, the unique N- and C-terminal regions of Rad may co-regulate CaM interaction. It is possible, of course, that deletion of the N terminus results in a more generalized alteration in conformation, exposing the CaM-binding site or altering the guanine nucleotide binding characteristics of this protein. In addition, accurate quantitation of the CaM binding efficiency of the N-terminal deletion mutant (Rad N88) is difficult since so little of the protein is detectable in the soluble lysate samples.

Unlike Ras, which is localized to the plasma membrane via prenylation of its C-terminal CAAX-like motif, Rad is localized mainly to the cytoplasm, with portions of the protein associated with cytoskeletal and membrane fractions (5). Although Rad lacks a CAAX-like C-terminal motif, deletion of the C-terminal residues 278–308 displaced Rad from the cytoskeleton, membrane skeleton, and soluble membrane fractions to the cytosol, whereas deletion of residues 297–308 did not. Thus, the critical residues for Rad localization to membrane and cytoskeletal components correspond to the CaM-binding domain of Rad, residues 278–297. It is also possible that CaM binding serves to localize Rad. Consistent with this, deletion of the N terminus of Rad, which enhances CaM binding, correlates with displacement of Rad from the cytosol to the cytoskeleton, membrane skeleton, and soluble membranes. Alternatively, in addition to an intact N terminus, Rad localization may require residues near, but distinct from, those C-terminal residues required for CaM interaction.

In summary, we have shown that the Ras-like GTPases, Rad and Gem, possess the unique quality of binding Ca\textsuperscript{2+}-CaM and have localized the site of CaM binding to the C-terminal residues 278–297 of human Rad. The interaction of Rad and CaM is dependent on the guanine nucleotide bound state of Rad, and deletion mutations that affect binding result in redistribution of Rad in the cell. In addition, Rad is found in complex with CaMKII, and Rad and Gem serve as in vitro substrates for this kinase, suggesting that the Rad-like GTPases participate in Ca\textsuperscript{2+}-activated signaling cascades leading to the activation of serine kinases.

Acknowledgments—We thank Dr. Renee Emkey for valuable discussions on this work. We also thank Dr. Kathleen Kelly of National Institutes of Health for the gifts of the GST-Gem construct and the anti-Gem antibodies.

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J. Biol. Chem. 1997, 272:11832-11839.
doi: 10.1074/jbc.272.18.11832

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