

Rad, a Novel Ras-related GTPase, Interacts with Skeletal Muscle β -Tropomyosin*

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Rad, a prototypic member of a subfamily of Ras-related GTPases, is overexpressed in skeletal muscle of type II diabetic humans. By expression screening of mouse embryo and human skeletal muscle cDNA libraries, we found that Rad interacted with skeletal muscle β -tropomyosin. In the mouse skeletal muscle cell line C₂C₁₂, this interaction was significantly increased by the calcium ionophore A23187. A23187 also caused a time- and concentration-dependent decrease in total cellular Rad with increased interaction between tropomyosin and Rad in the detergent-soluble fraction and the appearance of Rad in the cytoskeleton. In C₂C₁₂ cells stably overexpressing a putative dominant negative mutant of Rad (S105N), there was an increase in the amount of tropomyosin in Rad immunoprecipitates. In cells overexpressing wild type Rad, much of Rad was associated with the cytoskeleton and was no longer responsive to A23187. In far-Western blotting and guanine nucleotide saturation studies, GDP-Rad bound to tropomyosin far better than GTP-Rad. We conclude that Rad interacts with skeletal muscle β -tropomyosin and the cytoskeleton in a guanine nucleotide-dependent manner. These data suggest that Rad may be involved in skeletal muscle motor function and cytoskeletal organization.

Rad is the prototype of a growing subfamily of Ras-related GTPases with distinct amino and carboxyl termini. It was initially identified by subtractive cloning as a gene product overexpressed in the skeletal muscle of humans with type II diabetes (1). In normal humans, it is highly expressed in skeletal muscle, lung, and heart. It has a unique magnesium dependence with regard to guanine nucleotide binding and is a target of a cellular serine/threonine protein kinase (2). Evidence also suggests the existence of Rad-specific GTPase-activating protein (GAP).¹ Another Ras-related protein, Gem, shares significant sequence homology with Rad and is a mitogen-induced immediate early gene product in T lymphocytes (3). Kir is probably an alternatively spliced form of Gem, and its

expression is inducible by oncogenic tyrosine kinases in pre-B cells (4).

Evidence suggests that many members of the Ras GTPase superfamily play very diversified roles within the cells. Some of them are involved in normal cell functions such as cell signaling (5), growth and differentiation (6), vesicular transport (7), and nucleoprotein import (8). Recently, it has also been suggested that some play a role in cytoskeleton arrangement (9). For example, Rho has been shown to regulate the formation of actin stress fibers (10). Very recently, a direct interaction between Rho family members (Rac1, RhoA, and CDC42) and a novel myosin, myr 5, has been described *in vitro* (11). Although this has not yet been demonstrated *in vivo*, myr 5 appears to promote GTP hydrolysis by these Rho-related GTPases.

Since the cloning of *rad*, we have begun a series of studies in an attempt to determine the role of Rad in normal cell function. Rad is readily phosphorylated by the catalytic subunit of protein kinase A at the carboxyl terminus (2), enabling us to use phosphorylated Rad as a probe to screen expression libraries for potential Rad-associated proteins, similar to the approach used to identify the Ran-associated protein, RanBP1 (12). By screening both mouse embryo and human skeletal muscle expression libraries, we found that Rad interacted with skeletal muscle β -tropomyosin. This study reports the results of that screening and demonstrates that this interaction occurs in intact cells, is guanine nucleotide-dependent, and may be regulated by changes in calcium flux.

EXPERIMENTAL PROCEDURES

Cell Culture—C₂C₁₂ myoblasts (ATCC), a mouse skeletal muscle cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO₂ (13). Three to four days after reaching confluence, the medium was switched to DMEM containing 1% calf serum to facilitate formation of myotubes. After 5 days, approximately 80% of the culture was in the form of myotubes.

Expression Screening—A 16-day mouse embryo cDNA library in the λ EXlox vector (Novagen) was initially screened for potential Rad-associated proteins. Plating, manipulation of the library cDNA, and basic screening followed the supplier's recommendations and other published procedures (12). The library was plated at 40,000 plaques/plate (150 cm) in *Escherichia coli* strain BL21(DE3)pLysE. After 8 h, plates were covered with nitrocellulose filters (Schleicher & Schuell) impregnated with 10 mM isopropyl- β -D-thiogalactoside and incubated at 37 °C for another 14 h. Filters were washed four times for 10 min each with washing buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20, 10 mM β -mercaptoethanol). After a 4–6-h incubation at 4 °C in blocking buffer A (10 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5% dry milk, and 10 mM β -mercaptoethanol), filters were probed with [³²P]glutathione S-transferase-Rad (GST-Rad) overnight at 4 °C in blocking buffer B (blocking buffer A plus 200-fold molar excess of GST protein, 50 μ M GTP, 50 μ M GDP, and 5 mM MgCl₂). Filters were washed three times for 10 min each with washing buffer B (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 500 μ M GTP, 500 μ M GDP, 5 mM MgCl₂, 0.01% Tween 20, and 10 mM β -mercaptoethanol), air-dried, and exposed to X-Omat AR film (Eastman Kodak Co.). Potentially positive plaques were selected for secondary and tertiary screenings. The positive plaques were isolated,

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¹ The abbreviations used are: GAP, GTPase-activating protein; FBS, fetal bovine serum; GST, glutathione-S-transferase; DMEM, Dulbecco's modified Eagle's medium; GTP γ S, guanosine 5'-3-O-(thio)triphosphate.

and plasmids containing library cDNA were obtained by *in vivo* CRE-mediated recombination in *E. coli* strain BM25.8. Plasmids were transformed into XL1-Blue supercompetent cells (Stratagene) from which plasmids were retrieved, and DNA sequences were obtained using an Applied Biosystems automated sequencer. The same protocol was used to screen a human skeletal muscle cDNA library (see below).

Construction of Human Skeletal Muscle cDNA Library—The Poly(A)Ttract mRNA isolation system II (Promega) was used to isolate polyadenylated RNA from a sample of human skeletal muscle RNA (Clontech). Double stranded cDNA was made using the RiboClone cDNA synthesis system M-MLV(H⁻) (Promega) and was primed with oligo(dT). Internal *EcoRI* restriction sites were methylated according to the Promega Protocols and Applications Guide.

The cDNA was cloned into the λ EXlox vector (Novagen). The cDNA and *EcoRI* *I/HindIII* directional linkers were phosphorylated with T4 polynucleotide kinase. The linkers were then ligated to the cDNA via blunt end ligation. The *HindIII* and *EcoRI* sites were then cleaved with their respective restriction enzymes. The library was phenol/chloroform-extracted and size-fractionated using Sephacryl S-400, which isolates double-stranded DNA of ~300 bp and larger. After an ethanol precipitation, the cDNA was resuspended in 20 ml of TE. An aliquot of 3 ml of the cDNA was ligated to 0.5 mg of λ EXlox vector arms, and the DNA was packaged into virus using Novagen's packaging extract. This yielded a library with a titer of $\sim 2.0 \times 10^5$ plaque-forming units/ml.

Labeling of GST-Rad—Twenty μ g of GST-Rad prepared as described previously (2) was incubated with 100 units/2 μ g of catalytic subunit of protein kinase A (Sigma) and 1 mCi of [γ -³²P]ATP (DuPont NEN) in phosphorylation buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, and 1 mg/ml bovine serum albumin) at 37 °C for 15 min. [³²P]GST-Rad was separated from free nucleotide by passing the samples through two sequential NAP 10 gel filtration columns (Pharmacia Biotech Inc.). [³²P]GST-Rad was eluted with the phosphorylation buffer and incubated with 0.5 mM GTP, 0.5 mM GDP at room temperature for 15 min. The probe was diluted into blocking buffer B at approximately $2\text{--}4 \times 10^6$ cpm/ml.

Retroviral Transfection of Full-length *rad* cDNA—The full-length *rad* cDNA,² isolated previously from a diabetic subtraction library (1), was subcloned as a 1.4-kilobase pair fragment into the *BamHI-EcoRI* sites of the pBabe-Puro retroviral expression vector (14). A potential dominant negative mutant of Rad, devoid of GTP-binding activity *in vitro* (2), was created by converting Ser¹⁰⁵ to Asn (S105N).² The viral expression vector encoding this mutant was constructed by ligating a 453-base pair *Pf*MI-*EcoRI* fragment of the coding region of *rad*, which contained the mutation into the *Pf*MI-*EcoRI* sites of the wild type pBabe-*rad*. This resulted in an in-frame hybrid; the presence of the mutation was confirmed by DNA sequencing.

The BOSC23 packaging cell line, a gift from D. Baltimore, was maintained in DMEM containing 10% FBS at 37 °C in a humidified atmosphere of 10% CO₂, as described previously (15). Cells were transfected by calcium phosphate co-precipitation with 10 μ g each of the plasmids. Transiently produced viral supernatants were used to infect C₂C₁₂ cells in the presence of 4 μ g/ml Polybrene. After elimination of uninfected cells by puromycin, stable cell lines were maintained in DMEM containing 10% FBS. The expression levels were determined by immunoblotting with an antibody (JD68) generated against a fusion protein of GST with amino acid residues 40–308 of Rad (starting from the second in-frame ATG of *rad*). Expression of wild type Rad and mutant Rad were 30–40-fold and 20–30-fold, respectively, over that of endogenous Rad.

Immunoprecipitation and Immunoblotting—Human skeletal muscle proteins were extracted as described previously (2). C₂C₁₂ myotubes were treated at 37 °C with different reagents at the concentrations and time points indicated in the figure legends. Cells were solubilized in 1 ml of harvest buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 1 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 10 μ g/ml leupeptin, 1% Nonidet P-40, and 0.1% SDS). After solubilization at 4 °C for 60 min, samples were microcentrifuged at 4 °C for 15 min. The pellets (detergent-insoluble) were washed three times with the same buffer and saved for direct immunoblotting. Aliquots of 45 μ l (approximately 90 μ g of total protein) were withdrawn from the supernatant (detergent-soluble) for direct blotting, and the rest of the sample (approximately 2 mg of total protein) was subjected to immu-

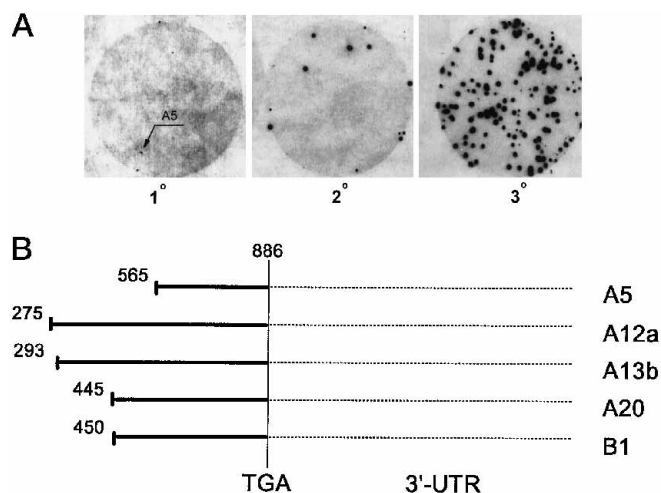


FIG. 1. Rad recognizes skeletal muscle β -tropomyosin in the expression libraries. A, GST-Rad was phosphorylated by protein kinase A and used to probe a 16-day mouse embryo cDNA library and a human skeletal muscle cDNA library. Protein expression was induced by incubation of plates with nitrocellulose filters impregnated in 10 mM isopropyl- β -D-thiogalactoside. Filters were washed, blocked, probed with [³²P]Rad-GST at 4 °C overnight, and washed again. Autoradiograms of the primary, secondary, and tertiary filter lifts of one of the positive plaques in mouse embryo library are shown. B, schematic representation of mouse skeletal muscle β -tropomyosin cDNA sequences isolated from the mouse embryo library.

noprecipitation with anti-Rad antiserum or anti-tropomyosin antiserum (Sigma). The final dilution for both antibodies was 1:100. Proteins were resolved on 10% SDS-PAGE, blotted to nitrocellulose filters (Schleicher & Schuell), and probed with antibodies as indicated.

Far-Western Blotting—Rabbit skeletal muscle tropomyosin (kindly provided by Dr. Lehrer of Boston Biomedical Research Institute) was resolved on 10% SDS-PAGE and blotted to nitrocellulose filters. Blocking, probing, and washings were carried out as for library screening except that after separation from free [γ -³²P]ATP on NAP 10 columns, [³²P]GST-Rad was incubated with 0.5 mM of either GTP γ S or GDP at room temperature for 10 min and then diluted into 5 ml of blocking buffer containing either of the nucleotides.

RESULTS

Rad Interacted with Skeletal Muscle β -Tropomyosin—Primary screening of a mouse embryo expression library using the human Rad revealed multiple candidate clones, which were subjected to secondary and tertiary screenings. In one typical trial, 118 potentially positive clones were isolated from 1.6×10^6 clones. All the clones were subjected to secondary screening, and 21 of these continued to be potentially positive. Tertiary screening at two dilutions confirmed five positive clones that came from different primary plates (Fig. 1A). Sequence analysis revealed that all five cDNA clones were 3'-end fragments of mouse skeletal muscle β -tropomyosin of different lengths. Since skeletal muscle is a rich source for Rad, we constructed a human skeletal muscle cDNA library using the same vector. Screening of 2×10^5 clones revealed six additional positives, all of which were shown to be human skeletal muscle β -tropomyosin by DNA sequencing. Thus in both mouse embryo and human skeletal muscle libraries, Rad interacted strongly with β -tropomyosin. The domain of tropomyosin that accounted for the interaction with Rad appears to be the COOH-terminal 96 or 107 amino acid residues in human and mouse β -tropomyosin proteins (Fig. 1B).

Tropomyosin Co-immunoprecipitated with Anti-Rad Antibody in C₂C₁₂ Muscle Cells—To determine if Rad interacts with tropomyosin in the context of the cellular environment, the C₂C₁₂ mouse skeletal muscle cell line was used. Myotubes were incubated with 10% FBS or calcium ionophore A23187 and solubilized in 1% Nonidet P-40 and 0.1% SDS. Direct immuno-

² The first in-frame ATG of *rad* is utilized *in vivo*. Hence, full-length Rad comprises 308 amino acid residues. The potentially dominant negative mutant of Rad (previously termed S66N-Rad) is therefore S105N-Rad.

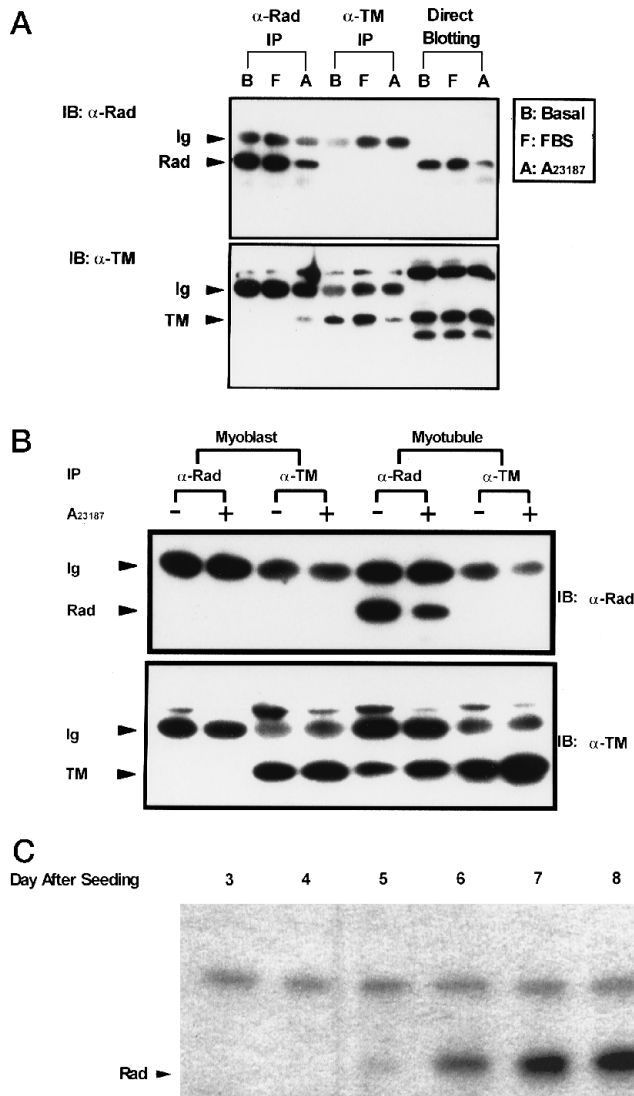


FIG. 2. Tropomyosin co-immunoprecipitates with Rad. C_2C_{12} cells were grown to confluence. Cell differentiation into myotubes was induced by culture in DMEM containing low serum. Cells were serum-starved overnight and treated with different agents, washed with cold PBS, and lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzimidazole, 10 μ g/ml leupeptin, 1% Nonidet P-40, and 0.1% SDS. After 1 h solubilization at 4 °C, detergent-soluble and insoluble fractions were separated by centrifugation in a microfuge for 15 min. The supernatant was divided and subjected to immunoprecipitation with anti-Rad or anti-tropomyosin antibodies. Immunoprecipitates were washed and further divided, resolved by 10% SDS-PAGE, and blotted with either of the antibodies. **A**, cells were treated with the Me_2SO vehicle, 10% FBS plus Me_2SO for 5 min, or 10 μ M A23187 for 18 min at 37 °C. For direct blotting, an aliquot of 45 μ l of the detergent-soluble supernatant (out of 1 ml) was withdrawn and resolved by 10% SDS-PAGE for Western blotting. **B**, myoblasts were frozen in the lysis buffer at -70 °C just before confluence and processed together with myotubes when the latter were available. Protein concentrations were normalized. Cells were either treated with Me_2SO or 10 μ M A23187 for 18 min at 37 °C. Samples were processed as in **A**. **C**, cells were seeded on day 0 at a density of $6 \times 10^5/10$ -cm dish, allowed to grow, and taken daily to prepare cell lysates as stated in Fig. 2. Protein concentrations were normalized and directly subjected to Western blotting. The positions of immunoglobulin (Ig), Rad, and tropomyosin are indicated.

blotting and blotting of the immunoprecipitates with a polyclonal Rad antibody identified a species of about 39 kDa representing endogenous Rad (Fig. 2A). Treatment of cells with 10% FBS did not change either the amount of immunoprecipitable Rad or total soluble Rad, whereas treatment with the calcium ionophore A23187 reduced both. Furthermore, upon

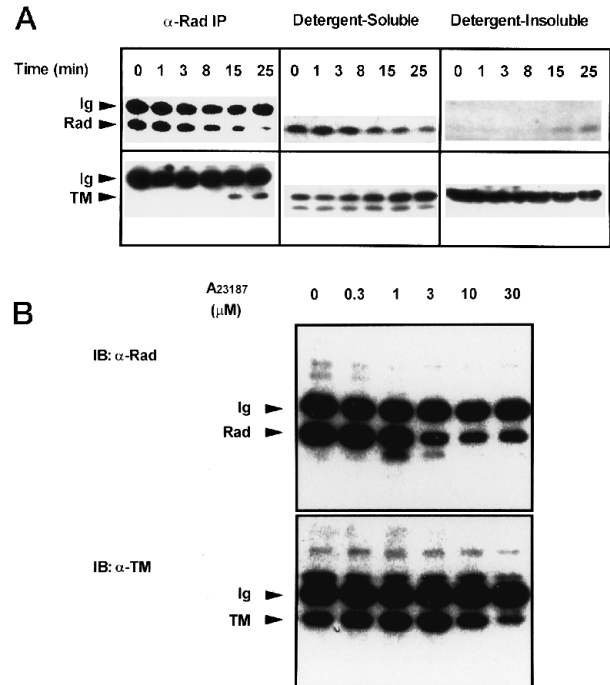


FIG. 3. A23187 causes translocation of Rad to the cytoskeleton, a decrease in total cellular Rad content, and an increase in association of Rad with tropomyosin in a time- and dose-dependent manner. C_2C_{12} myotubes were treated with Me_2SO or 10 μ M A23187 for indicated times (**panel A**) or 18 min with various A23187 concentrations (**panel B**) at 37 °C. Cells were lysed and samples were subjected to immunoprecipitation or direct blotting as described in Fig. 2. The positions of immunoglobulin (Ig), Rad, and tropomyosin are indicated.

A23187 stimulation, Rad immunoprecipitates also contained an increasing amount of tropomyosin, as seen on the tropomyosin immunoblot (Fig. 2A). By contrast, the tropomyosin antibody failed to co-precipitate detectable levels of Rad. This could be due to the much greater abundance of tropomyosin with respect to Rad in the skeletal muscle cells, such that when a small fraction of total cellular tropomyosin was immunoprecipitated, the associated Rad was below the limit of detection. Alternatively, the epitope on tropomyosin recognized by the antibody may have been masked by its interaction with Rad.

Expression of Rad and its association with tropomyosin was also dependent on the state of differentiation of the cells. Thus, Rad was virtually undetectable by immunoprecipitation in C_2C_{12} myoblasts in which tropomyosin was already expressed in appreciable quantities (Fig. 2B). Only in myotubes did association of tropomyosin with Rad occur, and this interaction was increased by A23187 treatment. Direct immunoblotting of C_2C_{12} cells at different stages of differentiation showed no detectable Rad in myoblasts, but Rad expression was elevated beginning on day 1 of differentiation (Fig. 2C). Therefore, it appeared that the expression of Rad increased with the extent of cell differentiation and that increased calcium influx caused a decrease in cellular Rad and an increase in the association between tropomyosin and Rad.

A23187 Stimulated Translocation of Rad to Detergent-insoluble Fraction—To better understand the effect of calcium ionophore on Rad content, Rad translocation, and its association with tropomyosin, the time course and dose response of the A23187 treatment were studied. The decrease in immunoprecipitable and total detergent-soluble Rad by A23187 treatment was time-dependent (Fig. 3A). A significant decrease in Rad was seen in Rad immunoprecipitates with prolonged A23187 treatment, while simultaneously there was increased Rad in

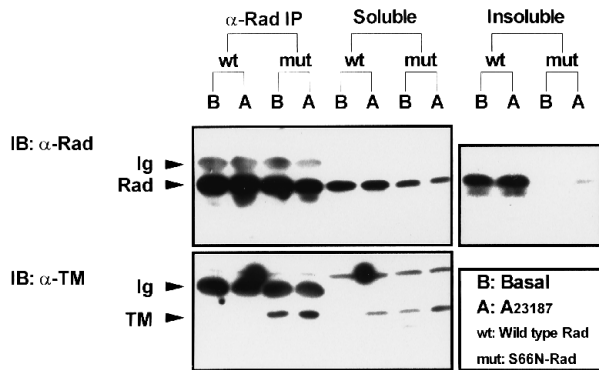


FIG. 4. **GDP-Rad associates with detergent-soluble tropomyosin and GTP-Rad interacts with cytoskeleton.** Wild type Rad and the potential dominant negative mutant of Rad (S105N) were overexpressed in C_2C_{12} cells by retroviral infection as described under "Experimental Procedures." Myotubes were treated with Me_2SO or $10 \mu\text{M}$ A23187 for 18 min, and samples were processed as described in Fig. 2.

detergent-insoluble fractions. The former could not be explained solely by translocation of Rad because the increase in detergent-insoluble fraction-associated Rad could not account for the loss of total detergent-soluble Rad (note that in the blots shown in Fig. 3, 10 times as much of the insoluble fraction were used for blotting). The increase in Rad-associated tropomyosin after A23187 treatment was time-dependent and occurred despite the decrease in Rad in the anti-Rad immunoprecipitates (Fig. 3A). A23187 also caused a decrease in immunoprecipitable Rad while increasing Rad-associated tropomyosin in a dose-dependent fashion (Fig. 3B). The half-maximal effect concentration of A23187 seemed to fall between 1 and $3 \mu\text{M}$ under the conditions used for treatment.

Association of Rad with Tropomyosin Was Guanine Nucleotide-dependent *In Vivo*—Using retroviral vectors, C_2C_{12} cells were stably transfected with wild type Rad or the S105N mutant of Rad, which is devoid of GTP-binding activity, but still binds GDP *in vitro*. There were no differences among parental cells and cells expressing wild type Rad or the mutant Rad in terms of gross morphology and ability to undergo differentiation. However, much larger amounts of tropomyosin were co-immunoprecipitated with Rad in cells overexpressing the S105N mutant than that in parental cells or cells overexpressing wild type Rad (Fig. 4). Although less prominent than was seen with parental cells (not shown), this association was still increased by A23187 treatment. Interestingly, a great amount of Rad was found in association with the detergent-insoluble fraction in the wild type Rad overexpressor, which was no longer stimutable with A23187. By contrast, cells that overexpressed mutant Rad had low levels of Rad associated with this fraction, which was fully stimutable with A23187. The latter probably reflected the level of endogenous Rad.

Tropomyosin Preferentially Bound GDP-Rad *In Vitro*—To further assess the requirement of guanine nucleotide binding for Rad-tropomyosin interaction, we performed far-Western blotting and *in vitro* co-precipitation assays. Purified rabbit skeletal muscle tropomyosin was resolved by SDS-PAGE and blotted to nitrocellulose filters. Rad was phosphorylated with protein kinase A *in vitro* followed by preincubation with either GDP or $\text{GTP}\gamma\text{S}$. GDP-loaded Rad strongly recognized tropomyosin, whereas $\text{GTP}\gamma\text{S}$ -loaded Rad weakly bound to it (Fig. 5).

Co-immunoprecipitation of tropomyosin with Rad was also obtained with human skeletal muscle (Fig. 6). Preincubation with GDP caused an increase in co-immunoprecipitation of tropomyosin with Rad, whereas preincubation with $\text{GTP}\gamma\text{S}$ failed to do so. Therefore, it is clear that in order for a strong

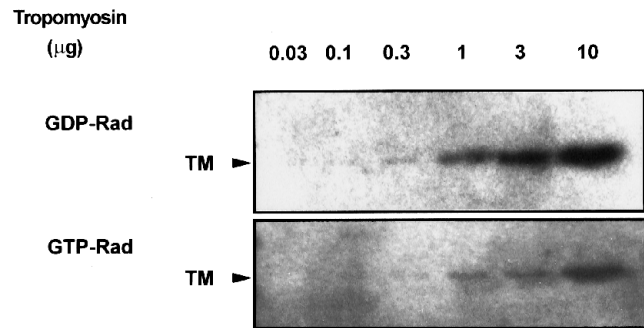


FIG. 5. **GDP-Rad binds tropomyosin more strongly than GTP-Rad on far-Western blotting.** Increasing amount of purified rabbit skeletal muscle tropomyosin were resolved in 10% SDS-PAGE, transferred to nitrocellulose filters, blocked, and probed with phosphorylated Rad as described for library screening under "Experimental Procedures" except that the probe (^{32}P Rad, $1.8 \mu\text{M}$) was equilibrated with either 0.5 mM GDP or 0.5 mM $\text{GTP}\gamma\text{S}$ at room temperature for 10 min before addition to the filters. After incubation at 4°C for 2 h, the filters were washed as described under "Experimental Procedures" and exposed to x-ray films.

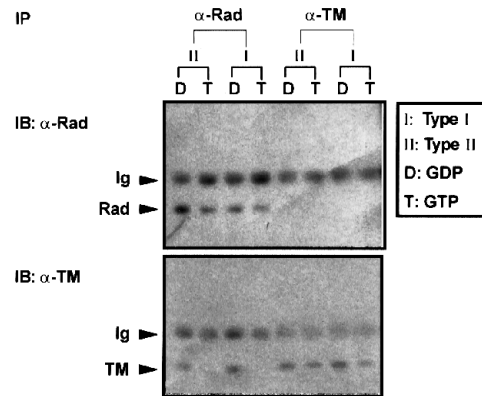


FIG. 6. **Tropomyosin binds GDP-Rad, but not GTP-Rad, in human skeletal muscle.** Skeletal muscle from type I and type II diabetic humans was homogenized with a Polytron homogenizer in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 1 mM MgCl_2 , and various protease inhibitors. Nonidet P-40 and SDS were added to final concentrations of 1% and 0.1%, respectively. After incubation at 4°C for 1 h, samples were centrifuged in a microcentrifuge for 15 min. The supernatant (0.5 mg of protein) was incubated with 0.5 mM GDP or GTP at room temperature for 15 min. Antibodies to Rad and tropomyosin were added for immunoprecipitation. Samples were processed as described in Fig. 2.

interaction to occur between tropomyosin and Rad, the GDP-rather than GTP-bound state of Rad is preferred.

DISCUSSION

Rad was the first member of a 35–39-kDa class of novel Ras-related GTPases, which includes Gem, Kir, and possibly other uncharacterized members. All of these GTPases possess highly conserved NH_2 -terminal extensions and COOH termini, which lack typical prenylation sites. They contain unique sequence variations in the G1 and G3 domains as compared to Ras, and novel G2 (effector) domains (2). Each was also identified in a specific context; Rad is increased in skeletal muscle of type II diabetes, whereas Gem and Kir are increased in activated T-lymphocytes and pre-B cells, respectively. The exact functions of these GTPases, however, remain unknown.

In order to understand the function of Rad in cells, we have searched for the proteins that interact with Rad by screening expression libraries. Using this approach, we find that Rad directly interacts with skeletal muscle β -tropomyosin. This interaction occurs using both intact cells and cDNA libraries from rodents and humans. Ninety-six amino acid residues of

the COOH terminus of skeletal muscle β -tropomyosin are sufficient to mediate the interaction with Rad. Tropomyosin is a classical molecule of α -helical coiled-coil with seven so-called α -repeats throughout its sequence (16). The COOH terminus of tropomyosin is important in mediating its binding to F-actin (17).

In the original report on the cloning of *rad*, we have shown that Rad is overexpressed in the skeletal muscle of type II diabetic humans, and that the highest expression in the normal humans is in skeletal muscle, heart, and lung (1). In rodents, heart and lung remain the highest expressors of *rad* mRNA, while skeletal muscle has somewhat lower level of expression.³ Thus, it is conceivable that skeletal and cardiac muscle are two of the major tissues in which Rad exerts its functions. Furthermore, although Rad is below the detection level in C₂C₁₂ myoblasts, its expression dramatically increases as the cells differentiate to myotubes, which suggests the regulation of Rad expression during myogenesis. Since the duration and extent of C₂C₁₂ cell differentiation are not altered by overexpression of either wild type or a putative dominant negative mutant of Rad, it is likely that Rad is not involved in the control of myogenesis, but rather that its expression is controlled along with or by myogenesis. Likewise, overexpression of Rad does not affect insulin- or serum-stimulated DNA synthesis or MAP kinase pathway, although it does reduce insulin-stimulated glucose uptake (18).

In light of the recent observation that a novel member of the myosin family, myr 5, possesses a GAP activity toward the Rho subfamily of Ras-like small GTPases (11), we have tested the effect of skeletal muscle tropomyosin on GTP binding to Rad, GDP dissociation from Rad, and the GTPase activity of Rad *in vitro*. No significant changes were observed; thus, under the conditions employed, skeletal muscle tropomyosin does not seem to have Rad GAP-like activity, nor does it act as a guanine nucleotide exchange factor for Rad (not shown). However, it is still possible that *in vivo*, additional molecules are needed to elaborate these specific effects.

The interaction between Rad and tropomyosin is highly regulated. In the basal state, a small fraction of Rad interacts with tropomyosin. This interaction is unaffected by insulin or serum but is increased by an elevated intracellular Ca²⁺ concentration induced by the calcium ionophore A23187. This is perhaps not surprising when we consider that skeletal muscle tropomyosin is involved in calcium-mediated muscle contraction. A23187 treatment also results in translocation of Rad to a detergent-insoluble fraction. This fraction is considered a cytoskeletal fraction (19). It is not known by which means and to which molecule Rad might attach; however, this interaction is extremely tight since it can withstand repeated washes in 0.5 M NaCl with 1% Nonidet P-40 and 0.1% SDS (data not shown). In platelets, Rap2B is translocated to the detergent-insoluble cytoskeleton upon thrombin stimulation, and it is therefore hypothesized that Rap2B may be involved in platelet activation (19). Similarly, translocation of Rad to cytoskeleton upon A23187 stimulation suggests a regulatory role for Rad in muscle contraction and/or cytoskeleton arrangement.

The second effect of A23187 treatment is a dramatic decrease in the cellular content of Rad in a relatively short period of time (estimated $t_{1/2} \leq 10$ min). This rapid decrease appears to be due to a rapid protein degradation mediated by calcium-sensitive proteases rather than relocation. The third effect of A23187 treatment is an increase in the association of Rad with detergent-soluble tropomyosin, despite the significant decrease in total Rad. The time course and dose dependence of this associ-

ation correlates with those of Rad translocation and degradation.

In order to further understand the association of Rad with tropomyosin, we expressed wild type and a putative dominant negative mutant of Rad in C₂C₁₂ cells. This mutant Rad (S105N) is analogous to the dominant negative mutant of Ras (S17N) and only binds GDP but not GTP. Interestingly, a large amount of tropomyosin is co-immunoprecipitated with Rad in cells overexpressing mutant Rad, whereas translocation of Rad to the cytoskeleton upon A23187 stimulation remains the same as in the parental cells. This strongly suggests that the detergent-soluble tropomyosin is associated with Rad when the latter is in a GDP-bound state. On the other hand, significant amounts of Rad are found in the detergent-insoluble cytoskeleton in cells overexpressing wild type Rad, and this association cannot be further stimulated with A23187. These observations have two implications. First, A23187 increases the number of Rad molecules in the GTP-bound state and translocation to cytoskeleton occurs. Second, there are a limited number of binding sites in the cytoskeleton for Rad such that, once they are occupied due to overexpressed Rad, Ca²⁺ influx is no longer able to stimulate further binding.

Further evidence for the dependence of Rad-tropomyosin interaction in the state of guanine nucleotide binding is provided by far-Western blotting and by immunoprecipitation of guanine nucleotide-saturated human skeletal muscle detergent extracts. It is clear that GDP-bound Rad associates with tropomyosin much more strongly than GTP-bound form. This difference is even more significant in human skeletal muscle when both Rad and tropomyosin are at their native conformations.

Thus, it is conceivable that skeletal muscle cells may have dual regulatory mechanisms with regard to Rad in response to calcium signal; a portion of Rad is translocated to cytoskeleton, where Rad may exert some as of yet unidentified function, *e.g.* involving the rearrangement of cell structure. Simultaneously, a proteolytic process is initiated, which may quickly reduce the effect of Rad in the cells. A well established mechanism by which activity of Ras-related small GTPases is terminated is their regulation by specific GAPs (20). The latter promote hydrolysis of GTP to GDP by GTPases, thus "switching-off" the GTPases. Besides this classical mode of regulation, rapid degradation may also serve as a "switching-off" mechanism. The finding that GDP-Rad binds to detergent-soluble tropomyosin upon A23187 stimulation suggests that tropomyosin can serve to sequester and compartmentalize Rad in its inactive GDP-bound form, thus providing a third possible means of "switching-off" the protein. The latter is similar to the reversible interaction between c-Jun and tropomyosin, which acts to sequester c-Jun in the cytoplasm (21), and to the Rab GDI that forms a complex with GDP-Rab, thus inhibiting the latter from binding to membranes (22). Alternatively, binding of GDP-Rad to tropomyosin may somehow regulate the functions of tropomyosin. The effect of Rad at different guanine nucleotide binding states on tropomyosin function is currently under investigation.

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