Journal of Cellular Biochemistry 65:527–541 (1997)

Identification of Rad's Effector-Binding Domain, Intracellular Localization, and Analysis of Expression in Pima Indians

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Abstract In order to characterize the endogenous gene product for rad (*ras*-related protein *a*ssociated with *d*iabetes), we prepared antibodies to synthetic peptides that correspond to amino acids (109–121, 178–195, 254–271) within the protein. These antibodies were used to analyze the expression, structure, and function of rad. Western analysis with these antibodies revealed that rad was a 46 kDa protein which was expressed during myotube formation. Further, immunolocalization studies showed that rad localized to thin filamentous regions in skeletal muscle. Interestingly, when muscle biopsies from diabetic and control Pima Indians were compared, no differences in rad protein or mRNA expression were observed. Similarly, no differences were observed in protein expression in diabetic and control Zucker diabetic fatty (ZDF) rats. Functional analysis of muscle rad revealed that its GTP-binding activity was inhibited by the addition of N-ethylmaliemide, GTP, GTPγS, and GDPβS but not ATP or dithiothreitol. Moreover, cytosol-dependent rad-GTPase activity was stimulated by the peptide corresponding to amino acids 109–121. Antibodies corresponding to this epitope inhibited cytosol-dependent rad-GTPase activity. Taken together, the results indicate that 1) rad is a 46 kDa GTP-binding protein localized to thin filaments in muscle and its expression increases during myoblast fusion, 2) expression of rad in Pima Indians and ZDF rats does not correlate with diabetes, and 3) the amino acids (109–121) may be involved in regulating rad-GTPase activity, perhaps by interacting with a cytosolic factor(s) regulating nucleotide exchange and/or hydrolysis. J. Cell. Biochem. 65:527–541.

Key words: insulin resistance; skeletal muscle; NIDDM; GTP-binding protein; thin filaments

Type II diabetes or NIDDM (non-insulin-dependent diabetes mellitus) is a serious metabolic disease resulting from an imbalance between insulin sensitivity and insulin secretion leading to its characteristic feature, hyperglycemia. Evidence exists implicating both environmental and genetic determinants as underlying causes of NIDDM [reviewed by Taylor, 1995]. In a few cases, the genetic basis of rare forms of NIDDM have been elucidated. For example, mutations in the genes for insulin, insulin receptor, glucokinase, and several mitochondrial proteins have been observed in some NIDDM patients [Turner et al., 1995].

Based on the findings that rad mRNA (*ras*-related protein *a*ssociated with *d*iabetes) was

Abbreviations used: GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GDPβS, guanosine-5′-O-(2-thiodiphosphate); GEF, guanine nucleotide-exchange factor; GTPγS, guanosine-5′-O-(3-thiotriphosphate); IDDM, insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; PMSF, phenylmethylsulfonyl fluoride; rad, *ras*-related protein associated with diabetes; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ZDF rat, Zucker diabetic fatty rat.

Contract grant sponsor: National Institute of Health, contract grant number DK 38765 and DK 47461; contract grant sponsor: Veterans Affairs Research Committee.

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Received 11 December 1996; accepted 12 February 1997

overexpressed in muscle from some type II diabetic patients, Rad was proposed to be a "candidate gene" involved in the pathogenesis of NIDDM and insulin resistance [Reynet et al., 1993]. Rad mRNA is expressed predominately in skeletal and cardiac muscle, suggesting rad may participate in signal transduction in muscle [Reynet and Kahn, 1993; Zhu et al., 1996]. Initial characterization studies of rad revealed that the recombinant form of the protein expressed in *E. coli* binds to and hydrolyzes GTP, indicating that nucleotide exchange and hydrolysis may be involved in its function [Zhu et al., 1995]. Although all of the studies to date on rad have been conducted using the recombinant form of the protein or its mRNA, no studies have been reported on the endogenous gene product for rad.

There are a number of cellular events mediated by the *ras*-related GTP-binding proteins. For instance, ras mediates cellular differentiation and growth, whereas the rho family of GTP-binding proteins is involved in cytoskeletal assembly [reviewed by Hall 1993], and the rab GTP-binding proteins are implicated in vesicular trafficking [reviewed by Nuoffer et al., 1994]. Since the cellular function of rad is unknown, understanding the regulation of its nucleotidase activity and its interaction with auxiliary protein(s) may provide further insight into its role within muscle.

Within the cell, the activity of the ras-related GTP-binding proteins is dictated by the regulation of bound GTP or GDP. The functional cycling of GTP/GDP is controlled by three classes of auxiliary proteins [reviewed by Bokoch and Der, 1993]. GTPase activating protein (GAP) and/or GDP dissociation inhibitor (GDI) increase the amount of protein in the GDP-bound state [McCormick, 1989; Boguski and McCormick, 1993; Takai et al., 1993]. Alternatively, guanine nucleotide-exchange factor (GEF) increases the amount of protein in the GTP-bound state [Jacquet et al., 1992].

In order to characterize the endogenous rad gene product, we made antibodies to synthetic peptides of rad (109–121 (I), 178–195 (II) and 254–271 [III]) that correspond to the "effector domain" (I), helix 3–loop 7 (II), and the "hypervariable region" (III) of *ras* [Pai, 1990], respectively. We chose these peptides because studies of chimeric proteins made from the ras-related proteins sec4 and YPT1 indicate that these regions confer specificity of function to GTP-binding proteins [Brennwald and Novick, 1993]. In the present study, we report for the first time on the subcellular and tissue distribution of the natural rad gene product and provide evidence that the expression of rad protein does not correlate with diabetes in either the Pima Indians or Zucker diabetic fatty (ZDF) rats. We also identify unique epitopes that are on the protein surface, characterize the GTP-binding properties, and identify a putative effector-binding domain within the endogenous muscle protein.

METHODS AND MATERIALS

Custom Peptide Synthesis and Antibody Production

Figure 1 shows the four peptides that were synthesized (Zeneca, Inc., Wilmington, DE) for these studies based on sequence alignments with ras. The rad amino acid sequence consisting of amino acids [109–121 (peptide I), 178–195 (peptide II), and 254–271 (peptide III)] correspond to the effector domain, helix 3–loop 7, and the hypervariable region of *ras* [Pai, 1990], respectively. These peptides (I–III) were used as antigens for generating polyclonal antibodies. Peptide IV (284–302) was used as a control for evaluating antibody specificity. Peptides I, II, and III were cross-linked to keyhole limpet hemocyanin antigen using standard chemistry with dithiobis-(sulfosuccinimidyl-propionate) (Pierce, Rockford, IL). Rabbits were immunized with 100 μg antigen every 2 weeks for 14 weeks, and the animals were exsanguinated on the fifteenth week. The antibodies were affinity-purified from serum using peptides cross-linked with dithiobis-(sulfosuccinimidyl-propionate) to a bovine serum albumin–sepharose column (Zeneca, Inc.).

Rad Analysis in Human Subjects

We studied insulin-sensitive and insulin-resistant Pima Indians with normal glucose tolerance and Pima Indians with non-insulin-dependent diabetes mellitus (NIDDM). Consent was obtained from all subjects participating in this study. Normal glucose-tolerant individuals were designated insulin-sensitive or -resistant based on maximal *in vivo* glucose uptake rates during hyperinsulinemic euglycemic clamp studies. Insulin-sensitive subjects had maximal glucose uptake >11.5 mg/kg lean body mass/min, and insulin-resistant individuals were <7.0 mg/kg lean body mass/min. The

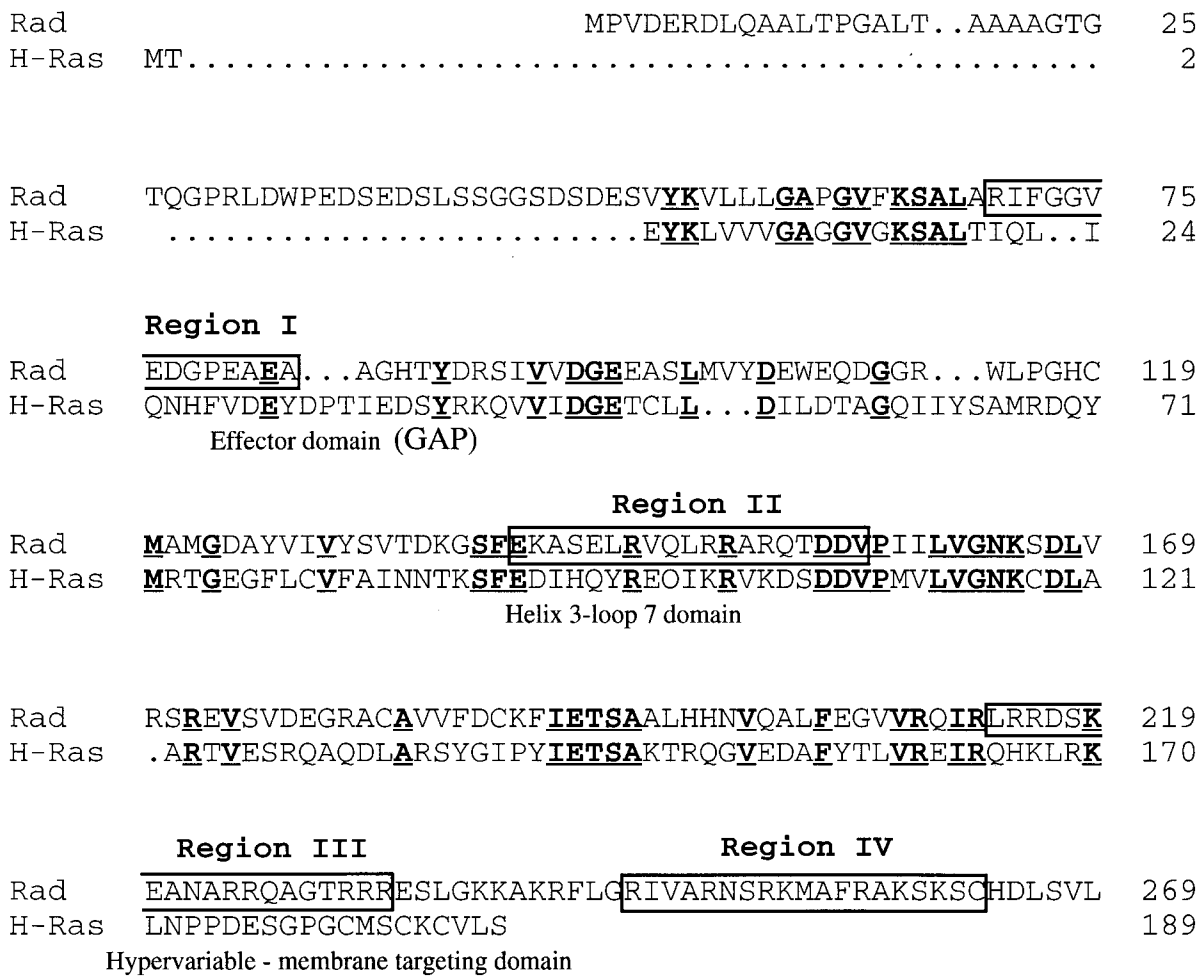

Fig. 1. Comparison of amino acid sequences of rad and h-*ras*. Three peptides from the rad sequence that correspond to the effector domain (region I), the helix, helix 3–loop 7 motif (region II), and the hypervariable region (region III) of ras were synthesized and used for antibody production as described in Methods and Materials (boxes). The *ras* sequence and its proposed domains are shown as reviewed by Marshall [1993]. Dots represent gaps inserted to allow for optimal alignment of the sequences.

methods for the glucose clamp technique and determination of body composition and lean body mass by underwater weighing were as previously described [Garvey et al., 1992]. NIDDM patients were not being treated with hypoglycemic agents at the time of study. Diagnosis of NIDDM was according to criteria of the National Diabetes Data Group [1979].

Percutaneous biopsies of the vastus lateralis were performed as previously described [Garvey et al., 1992]. In each subject, 100–200 mg of frozen (−80°C) muscle tissue was processed and fractionated as described previously [Garvey et al., 1992].

Cell Culture and Preparation of Cell Lysates

L6 rat myoblasts were plated at a density of 3×10^6 cells/well in 12 well Costar plates (Costar, Cambridge, MA). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated in 10% CO_2 at 37°C. For harvesting total cell lysates, we washed the cells three times with ice-cold phosphate-buffered saline and scraped them into lysis buffer consisting of 5 mM Tris-HCl, pH 7.7, 1 mM $MgSO_4$, 1 mM dithiothreitol, and 0.001% digitonin. The lysates were then processed for SDS-PAGE as described below.

Subcellular Fractionation

Hind-limb skeletal muscle from 8–10 week Sprague-Dawley or ZDF rats was suspended (20% w/v) in homogenization buffer (250 mM sucrose, 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 mM EGTA, 10 mM HEPES, pH 7.5) and homogenized for 30 s at 8,000 rpm/min (UT-T25 ho-

mogenizer knife; IKA, Cincinnati, OH) at 4°C. Cytosol and membranes were prepared as previously reported [Colombo et al., 1992]. In order to eliminate endogenous nucleotides, we twice concentrated the cytosol used for GTPase studies in a Centricon YM-10 (Amicon, Danvers, MA) microconcentrator for 2 h at 6,000*g* and reconstituted it to the original volume in phosphate-buffered saline. The protein concentration was determined using the BCA® reagent (Pierce) according to the manufacturer's protocol.

Immunolocalization

Frozen sections of skeletal muscle from normal adult Sprague-Dawley rats were cut to 3 µm and mounted on Super Frost Plus® slides (Fisher, Orangeburg, NY). Sections were fixed in acetone for 5 min and air-dried. Rabbit anti-tropomyosin polyclonal antibody (Chemicon, Temecula, CA) or rad-antibody II (+/− 1.0 µM rad peptide II) were applied at a 1:100 dilution in Automation Buffer™ (Biomeda, Foster City, CA) and incubated for 1 h at 22°C. The slides were rinsed with Automation Buffer™ and stained using either the Vectastain® ABC-GO (glucose oxidase) for rad or Vectastain® ABC-AP (alkaline phosphatase) immunohistochemical kit for tropomyosin (Vector Labs, Burlingame, CA) according to the manufacturer's specifications. Incubations which included the peptide antigen or secondary antibody alone were used as controls for nonspecific staining.

Western and Northern Blot Analysis

Western analysis. Human protein medleys (Clontech Laboratories, Palo Alto, CA) and ZDF rat skeletal muscle samples (50 µg of protein) were resolved under reducing conditions using 4–20% Tris-glycine precast polyacrylamide gels (Novex, San Diego, CA). Protein was transferred to nitrocellulose according to the manufacturer's specifications (Novex). In all instances, antigens were detected using the ECL reagent according to the manufacturer's protocol (Amersham, Arlington Heights, IL).

Northern analysis. Recombinant cDNA sequences including nucleotides 252–1,021 of Rad [Reynet and Kahn, 1993] were obtained using standard polymerase chain reaction techniques. A precast human multiple tissue Northern blot (Clontech) was prehybridized overnight at 42°C in 50% formamide, 5X SSC, 0.1% SDS, 1X Denhardt's solution, and 150 mg/ml sheared salmon sperm DNA. The blot was incubated with [^{32}P]-labeled Rad cDNA overnight at 42°C, washed at 55°C in 0.2X SSC/0.1% SDS, and exposed to Kodak XAR-5 film for 16 h at −80°C and processed in a Kodak autoprocessor.

RNA Extraction and Ribonuclease Protection Assay

Total RNA was extracted from snap-frozen muscle biopsy samples taken from insulin-sensitive, insulin-resistant, and NIDDM volunteers. Each sample was homogenized in 8.0 ml of RNAzol B (Tel-Test, Inc., Friendswood, TX), supernatants were precipitated with −20°C isopropanol, and the RNA pellets were washed with −20°C 75% ethanol. Each single-stranded antisense probe was hybridized overnight at 50°C with 2 µg of total RNA. Following hybridization, the samples were incubated for 30 min in a cocktail of RNase TI and RNase A to digest the unprotected RNA. The protected fragments were precipitated and separated on a 6% polyacrylamide denaturing gel, and the radioactive bands were analyzed on a PhosphorImaging system (Molecular Dynamics, Sunnyvale, CA). The Rad signal was normalized to the signal for the S20 ribosomal protein.

GTP-Binding and Hydrolysis Assays

GTP-Binding Assay. Costar 96 well plates were incubated with 200 µl of antibody (0.1 mg/ml in phosphate-buffered saline [PBS]) for 16 h at 4°C. After removal of the unbound antibody, the plates were incubated with blocking buffer (PBS, pH 7.2, 3% bovine serum albumin, 0.2% Tween-20) for 20 min at 22°C. The supernatant was removed, and the plates were incubated for 20 min at 22°C in blocking buffer, plus or minus peptide, ATP, GTPγS, GTPβS, or mastoparan at the specified concentrations. The supernatant was removed, and the plates were incubated for 1 h at 22°C with 200 µl/well of Sprague-Dawley rat skeletal muscle cytosol (100 µg) in PBS (pH 7.2), 100 µM PMSF, 1 µM pepstatin A, 10 µg/ml trypsin inhibitor, plus or minus peptide. The plates were rinsed three times with blocking buffer and incubated for 20 min at 22°C in 200 µl of buffer I (50 mM Tris (pH 7.5), 5 mM $MgCl_2$, 10 mM EDTA, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin) containing 10 µCi/ml [α^{32}P]GTP (3,000 Ci/mmol) (Amersham), plus or minus peptide, GTPγS, GTPβS, or ATP. The plates were rinsed three times with buffer I, and the protein was solubilized with 100 µl of 0.4 M NaOH. Radioac-

tivity was determined using a micro-beta plate reader (Wallac Inc., Gaithersburg, MD).

GTP-Hydrolysis Assay. Rad was immobilized onto 96 well plates as described for the GTP-binding assay. The GTP-hydrolysis assay was done using the buffer system described by others [Settleman et al., 1992]. GTP hydrolysis was measured by thin layer chromatography (TLC) as previously reported [Park et al., 1993]. The amounts of GDP and GTP were quantitated by scanning densitometry using the Bio-Rad Image System with the Molecular Analyst software (BioRad, Richmond, CA) and expressed as the ratio of [GDP]/[GTP + GDP].

RESULTS

Endogenous Rad Is a 46 kDa Protein Expressed Predominantly in Human Skeletal and Heart Muscle

It was unknown if expression of the gene product for rad correlates with expression of its mRNA. Thus, we looked for codistribution of rad protein and mRNA in various human tissues by Western and Northern analysis, respectively. First, in order to identify the endogenous rad gene product, we compared the peptide antibodies by Western blot analysis of human skeletal muscle. The results in Figure 2A show that antibodies I and II bind to a protein of the same molecular weight (46 kDa) present in muscle. Interestingly, immunostaining was not detected in the presence of antibody III, suggesting this epitope is not recognized under denaturing conditions (data not shown). Moreover, only the respective peptide for each antibody blocked binding to rad, indicating the antibodies are specific for each epitope (Fig. 2A). Next, we compared rad protein and mRNA expression in several human tissues. Rad protein (Fig. 2B) and mRNA (Fig. 2C) were detected mainly in heart and skeletal muscle and to a lesser degree in kidney. The protein and mRNA for rad were not detected in brain or liver (Fig. 2B,C). Taken together, these results indicate that rad is a 46 kDa protein expressed primarily in muscle and suggest that expression of the rad gene product correlates ($r = 0.76$) with expression of its mRNA.

RAD Expression in Human Skeletal Muscle Does Not Correlate With Type II Diabetes

Initial studies reported that rad mRNA expression was increased in skeletal muscle of Caucasians with NIDDM relative to normal and IDDM individuals [Reynet and Kahn, 1993]. However, studies on rad expression have not been reported in other diabetic populations (e.g., Pima Indians). Further, it has not been determined if the gene product for rad is overexpressed in NIDDM patients. Therefore, we examined both rad protein and mRNA levels in Pima Indians, a well-characterized population genetically predisposed to NIDDM.

We used nucleic acid probes and antibodies to evaluate the expression of rad mRNA and protein in human skeletal muscle biopsies from normal (glucose tolerant) and diabetic Pima Indians. The normal glucose-tolerant patients were further subdivided into insulin-sensitive and insulin-resistant subgroups. These divisions were based on results obtained during hyperinsulinemic euglycemic clamp studies which predominantly measure insulin-stimulated glucose uptake into skeletal muscle. The clinical characteristics of the Pima Indian study groups are shown in Table I. Ribonuclease protection assays (Fig. 3A) with subsequent quantitation (Fig. 3B) revealed no significant differences in rad muscle mRNA levels among the normal and diabetic subjects. In order to assess rad protein levels in these subjects, Western blot analysis was done on postnuclear homogenates of vastus lateralis biopsies. The results indicate that rad protein levels in muscle biopsies taken from these individuals were similar among insulin-sensitive, insulin-resistant, and NIDDM individuals (Fig. 4A,B).

As a second model to determine if rad protein expression correlated with NIDDM, we performed Western analysis for rad in skeletal muscle of ZDF rats ($n = 8$). The ZDF rat is a commonly used rodent model for investigating the biochemical consequences of diabetes [reviewed by Shafrir, 1992]. The expression of rad and actin (a positive control) was determined by scanning densitometry of the Western blots. There was no statistical difference in rad expression levels (normalized to actin) between normal (0.531 ± 0.026 units rad/actin) and diabetic animals (0.470 ± 0.018 units rad/actin).

Rad Localizes to Muscle Thin Filaments, and Its Expression Increases During Myotube Formation

In order to gain further insight into rad's potential function, we performed immunocytochemical analysis to determine the intracellular localization of rad in rodent skeletal muscle tissue. As shown in Figure 5A, rad antibody II stained thin filamentous structures in muscle.

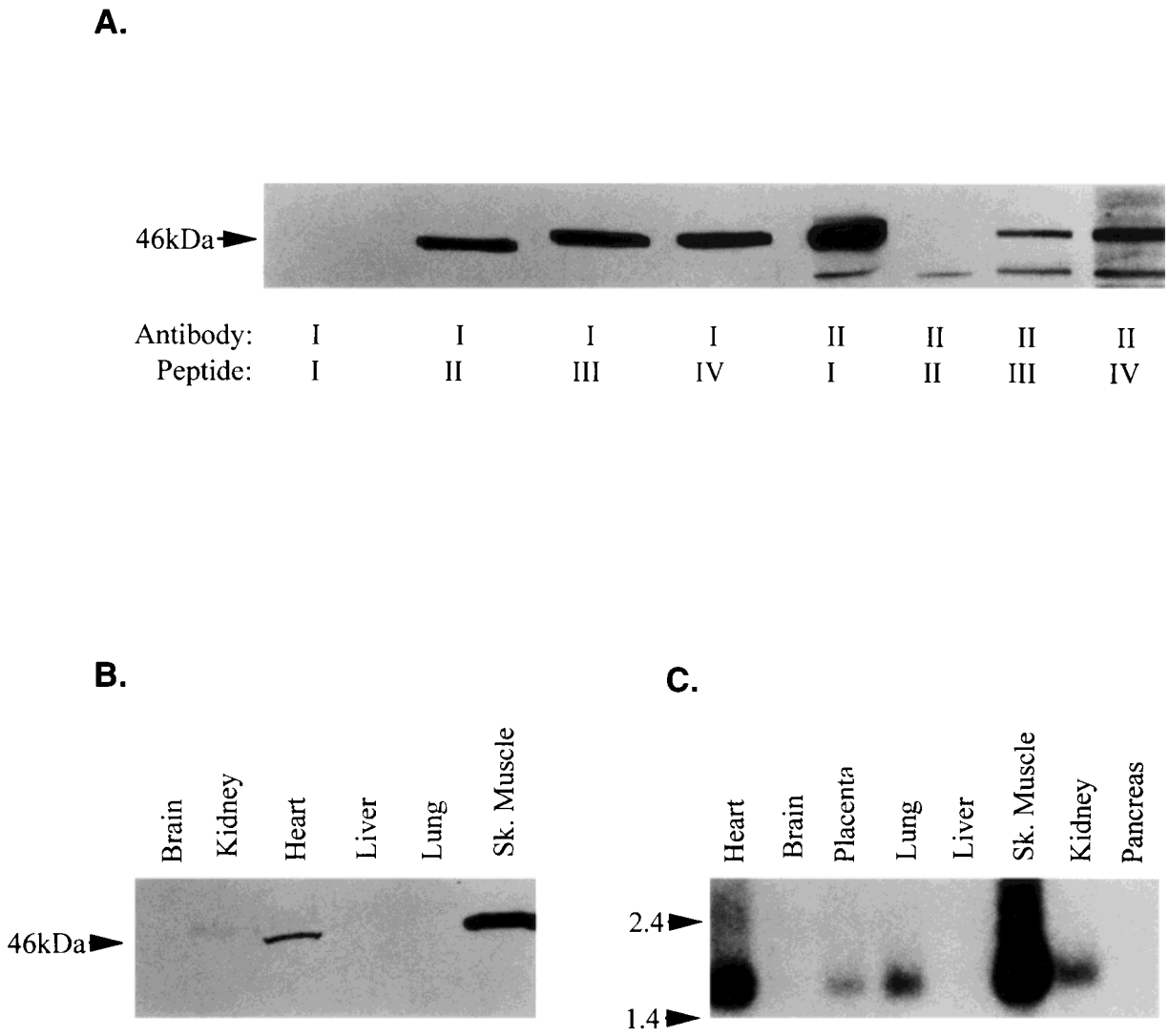

Fig. 2. Rad is a 46 kDa protein predominantly found in skeletal muscle and heart. **A:** Western blot analysis of human skeletal muscle protein was performed with antibodies to peptides I (*lanes 1–4*) and II (*lanes 5–8*). The 46 kDa protein was not observed when antibody I was incubated with 1.0 μM peptide I (lane 1), but was observed in the presence of peptides II (lane 2), III (lane 3), and IV (lane 4). Similarly, the 46 kDa protein was not observed when antibody II was incubated with 1.0 nM peptide II (lane 6) but was observed in the presence of peptides I (lane 5), III (lane 7), and IV (lane 8). **B:** Western blot analysis of various human tissues using rad antibody II. Equal amounts of protein (50 μg) were loaded in each lane. **C:** Northern blot analysis of polyA+ RNA from a variety of human tissues probed with a radiolabeled fragment corresponding to nucleotides 251–1,060 of rad. As an internal control, the rad signal was normalized to the signal for the S20 ribosomal protein.

No staining occurred in the absence of antibody II (secondary antibody alone) or when antibody II was incubated in the presence of peptide II (Fig. 5D,E). The same staining pattern as antibody II was observed using antibodies I or III, confirming rad is localized to filamentous regions (data not shown). To further characterize the localization of rad, we used antibodies to tropomyosin (a protein associated with thin filaments) [Bennett, 1989] either alone (Fig. 5B) or in conjunction with rad antibodies (Fig. 5C). The staining patterns show that rad colocalized with tropomyosin to thin filaments (comparing Fig. 5 A–C).

Since a number of cytoskeletal proteins in muscle are regulated during differentiation [Paulin et al., 1980], we looked at rad's expression during myotube formation. The expression of rad protein during myotube formation was determined by Western analysis of L6 rat myoblasts at various stages of differentiation (Fig. 6A,B). The results show that rad expression

TABLE I. Clinical Characteristics for the Subgroups of Pima Indians*

Parameter	Insulin-sensitive	Insulin-resistant	NIDDM
Age (years)	26 ± 2	30 ± 3	31 ± 2
Body mass index (kg/m^2)	27 ± 1	45 ± 3	43 ± 2
% Body fat	22 ± 1	37 ± 1	40 ± 3
Waist/thigh ratio	1.6 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Fasting plasma glucose (mM)	4.5 ± 0.1	5.0 ± 0.3	7.3 ± 1.4
Maximal glucose uptake (mg/kg/min)	14.3 ± 0.5	6.1 ± 0.2	5.6 ± 2.0

*Insulin-sensitive and insulin-resistant nondiabetic subjects were defined on the basis of maximally insulin-stimulated glucose disposal rate >11.5 or <7.0 mg/kg lean body mass/min, respectively. The methods for determination of body composition, lean body mass, and the glucose clamp technique were done as previously described [Garvey et al., 1992].

began to increase after 7 days and was maximal by 14 days of differentiation. Rad expression correlated well ($r = 0.96$) with the expression of the muscle marker protein, Myo D (data not shown), as well as the time required for formation of myotubes from myoblasts (10–14 days) [Lawrence and Coleman, 1984; Florini et al., 1984].

Structure and Functional Analysis of Endogenous Rad

Although the GTP-binding properties of recombinant rad have previously been reported [Zhu et al., 1995], the GTP-binding properties of endogenous rad from muscle have not been demonstrated. Moreover, the structural domains exposed on the surface of the protein, the requirement for sulfhydryl groups, and the sequences involved in protein-protein interactions have never been studied in either recombinant or endogenous rad. Thus, we used rad antibodies and their respective peptides to further explore some of the structural and functional characteristics of endogenous rad.

In order to test if the natural gene product for rad in rat skeletal muscle binds to GTP, we used a radioimmunoassay that detects binding of [α^{32}P]GTP-labeled protein to the rad-specific peptide antibodies. Incubation of antibody II–coated microtiter plates with [α^{32}P]GTP-labeled muscle extracts revealed that this antibody recognized a GTP-binding protein in muscle (Table II). [α^{32}P]GTP binding to the plate was inhibited by incubating antibody II with 10 μM peptide II but not peptides I, III, and IV (Table II). Similar results were obtained with antibody I and III (data not shown), indicating that these three epitopes represent exposed domains on rad. To confirm the specificity of nucleotide binding, we tested the effects of various nucleotides on GTP binding. As expected, GTP, GTPγS, and GDPβS but not ATP inhibited [α^{32}P]GTP binding to rad (Table II). We also tested the effects of mastoparan, a toxin that accelerates nucleotide exchange on some Gα proteins [Higashijima et al., 1990], on GTP binding. Mastoparan was without effect on rad's ability to bind GTP, indicating rad does not belong to the mastoparan-sensitive class of G proteins.

Three of the four cysteine residues (119, 182, and 188) in rad are located near the predicted nucleotide-binding domain, suggesting one or more of these residues, perhaps in a reduced form, might be critical for GTP binding. In order to determine the structural importance of these cysteines in maintaining proper protein function, both N-ethyl-maliemide (an alkylating agent that modifies cysteine residues) and dithiothreitol (a reducing agent) were tested for their effects on GTP binding. N-ethylmaliemide inhibited GTP binding, indicating sulfhydryl groups may be critical for ligand binding (Table II). Dithiothreitol slightly stimulated GTP binding (Table II). Although the stimulation by dithiothreitol is not statistically significant, this increase was observed in multiple experiments. When analyzed by Western blot after electrophoresis using reducing and nonreducing conditions, no difference in rad's molecular weight was observed (data not shown). This result suggests that rad may not form interdisulfide bonds. Antibodies I and III yielded similar results in these studies (data not shown).

We were interested in determining if rad interacts with auxiliary proteins that regulate GTPase activity (e.g., GAP, GDI, and/or GEF) and characterizing which domains may be involved in this interaction. Thus, we examined the effect of synthetic peptides and antibodies on rad's GTPase activity in the presence or absence of cytosol. As described in Materials and Methods, antibody II–coated plates were used to immobilize muscle rad prior to measuring GTPase activity. As expected, the addition

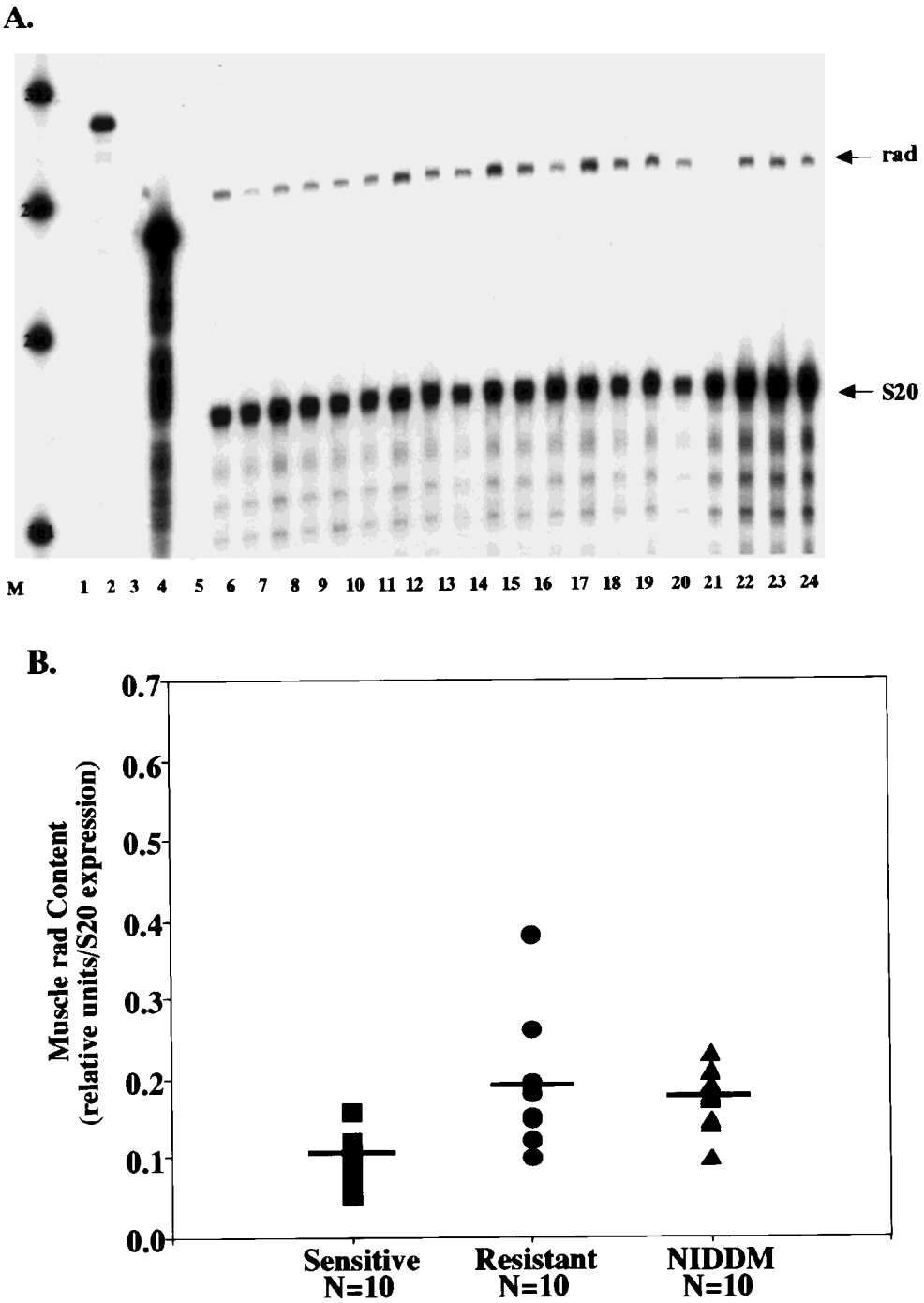

Fig. 3. Rad mRNA expression does not correlate with type II diabetes in Pima Indians. **A:** Ribonuclease protection assay of rad mRNA. A 288 base radiolabeled antisense riboprobe for detecting rad was prepared that included nucleotides 311–560 of the rad cDNA. The presence of rad mRNA is demonstrated by the presence of a 250 base protected band. Ribosomal protein S20 was chosen as an internal control because its mRNA expression does not appear to change with insulin resistance or diabetic state. The antisense riboprobe was 230 bases of which 173 bases are protected by S20 mRNA. Lanes 1 and 3 represent undigested Rad and S20 probes, respectively. Lanes 2 and 4 are digested Rad and S20 probes, respectively. The following lanes represent the various subjects biopsied: 5–9 (insulin-sensitive), 10–14 (insulin-resistant), 15–20 (non-insulin dependent diabetics). Lanes 21 and 22–23 represent liver and skeletal muscle RNA, respectively. Whereas, lane 24 is heart RNA. **B:** Quantitative analysis of rad mRNA levels in the various subjects tested. Rad mRNA levels were normalized to the ribosomal protein S20 levels in each tissue sample. Bars represent means of each group.

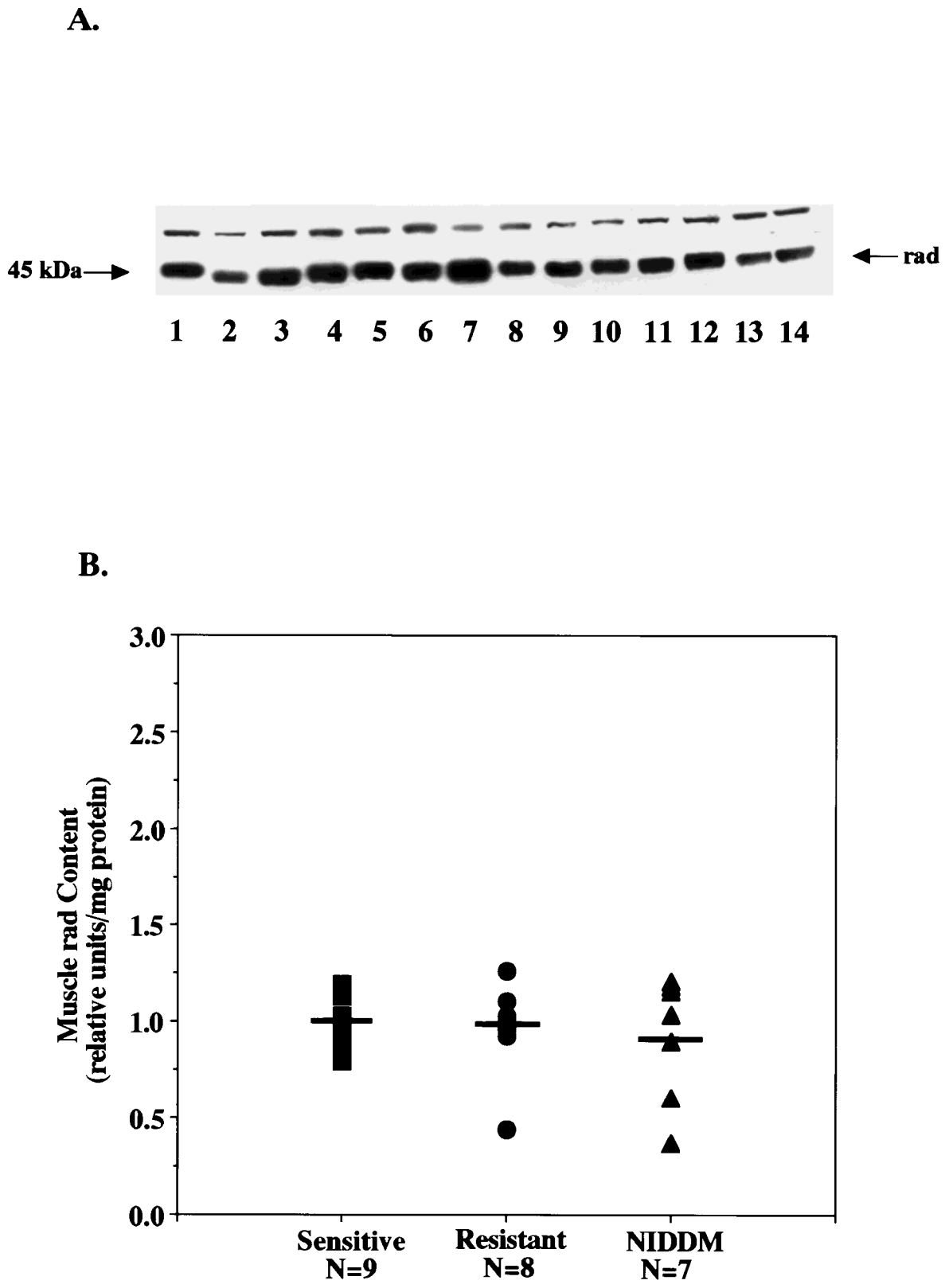

Fig. 4. Rad protein expression does not correlate with type-II diabetes in Pima Indians. **A:** Representative Western blot analysis for rad from biopsies of vastus lateralis obtained from insulin sensitive, insulin resistant, and NIDDM individuals. Rad migrated as a 46 kDa polypeptide in all subject groups. Lanes 1–5 (insulin sensitive), 6–10 (insulin resistant) and 11–14 (NIDDM) subjects. Equal amounts of protein (50 μg) were loaded in each lane. **B:** Quantitative analysis of rad protein measured in total post-nuclear homogenates of vastus lateralis biopsies. Relative levels were quantitated by densitometry and were normalized per mg of protein. Horizontal lines indicate mean values which are not statistically different among the subject groups.

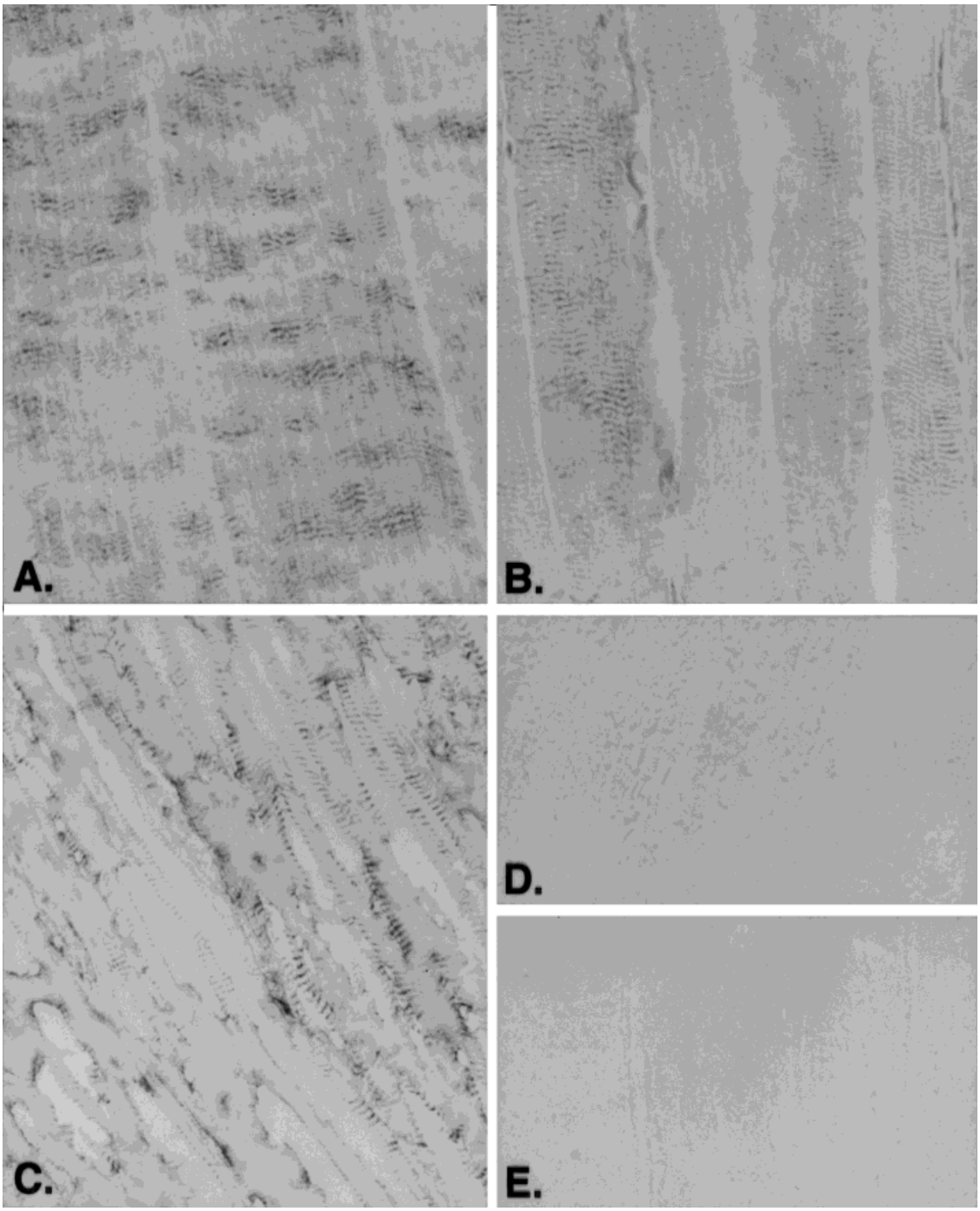

Fig. 5. Rad localizes to thin filaments in skeletal muscle. Skeletal muscle sections were incubated in the presence of **A:** rad antibody II, **B:** tropomyosin, **C:** rad antibody II and tropomyosin antibodies, **D:** rad antibody II plus peptide II antigen, and **E:** no primary antibody addition. The samples were prepared for immunocytochemistry as described in the Methods and Materials.

of peptide II prevented detection of GTPase activity (data not shown).

It is shown in Figure 7 that GTP hydrolysis by endogenous rad was potentiated by the addition of cytosol (Fig. 7A,B). When the peptides were tested for their effects on the cytosol-dependent rad GTPase activity, 1 μM peptide I (a.a. 109–121) potentiated GTP hydrolysis (74% stimulation) by rad. Peptide I had no effect in the absence of cytosol (Fig. 7A). Additionally,

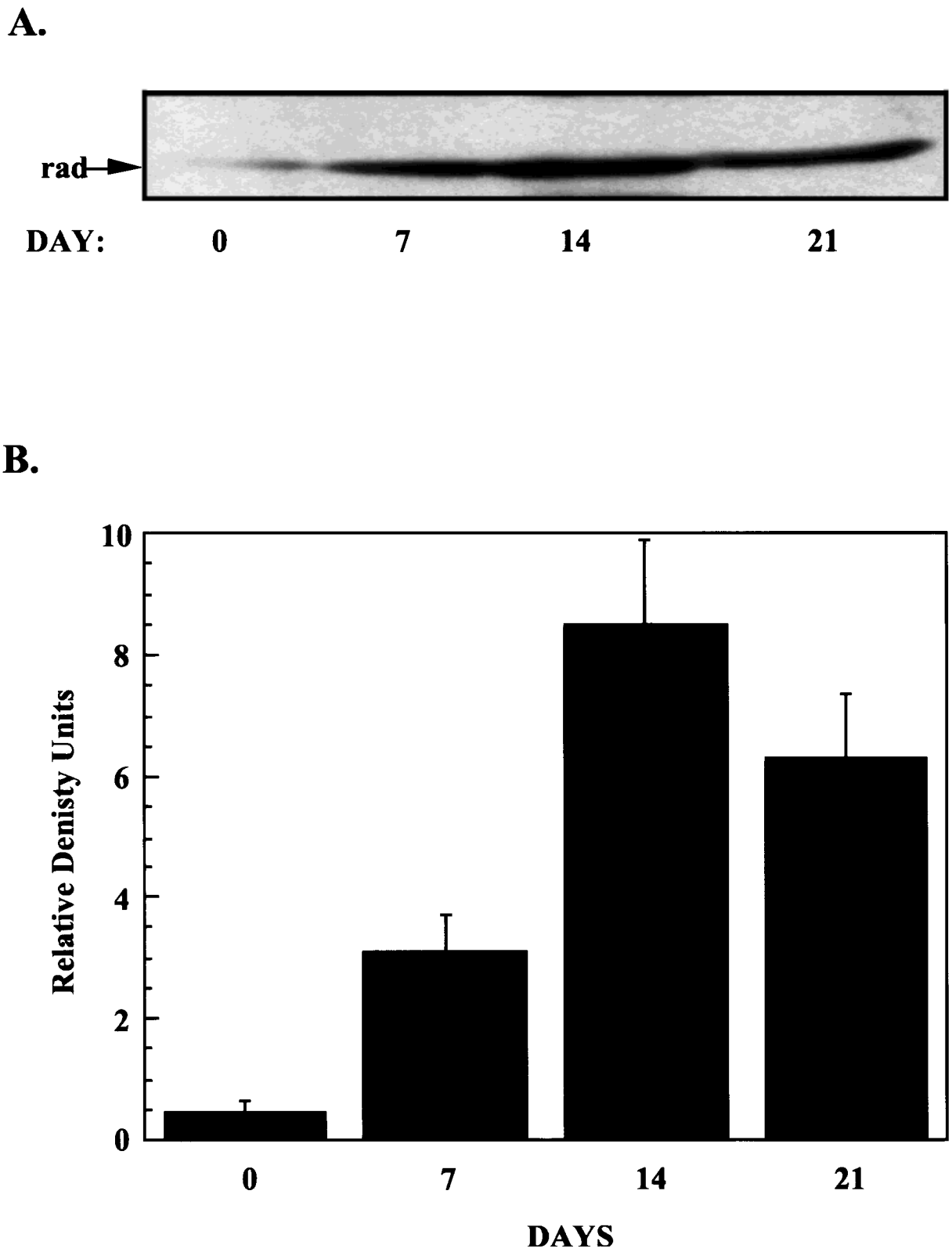

Fig. 6. Rad protein expression increases during myogenesis. **A:** Western blot analysis for rad from L6 myoblast cell lysates prepared after 0–14 days in culture. Equal amount of protein (12 μg) was loaded onto each lane. **B:** Quantitative analysis of rad immunoblots from L6 myoblasts at different stages of differentiation. Relative levels of rad protein expression were quantitated by densitometry and expressed as relative density units.

TABLE II. Effects of Various Agents on the [α^{32}P]GTP Binding Capacity of Rad

Experimental conditions	Concentration[a]	Relative binding (%)[b]
GTP	0.1	11.3 ± 2.1
Peptide II	0.01	11.8 ± 9.4
GTPγS	0.1	15.7 ± 2.4
GDPβS	0.1	18.8 ± 2.1
Mastoparan	0.01	76.5 ± 0.9
N-ethyl maleimide	0.001	87.9 ± 4.6
ATP	1.0	96.4 ± 4.4
Peptide I	0.01	96.6 ± 4.6
Peptide III	0.01	98.4 ± 15.4
No treatment	—	100.0 ± 2.2
Peptide IV	0.01	105.4 ± 8.9
Dithiothreitol	0.01	108.4 ± 8.5

[a]All concentrations are in millimolar amounts.
[b]The amount of [α^{32}P]GTP bound is expressed as a percent of relative binding compared to the no treatment control. The binding assay was performed in the absence or presence of the indicated agent (n = 3).

peptide III (a.a. 254–271) had no effect on GTP hydrolysis in the presence or absence of cytosol (Fig. 7A). This suggests that amino acids (109–120) may be involved in modulating cytosol-dependent rad-GTPase activity, possibly by mediating the interaction of an auxiliary factor(s) with rad. To further explore if amino acids (109–120) were important structural determinants in rad, the antibodies which were generated from the peptides were tested for their effects on GTPase activity. Figure 7B shows that antibody I inhibited (58% inhibition) the cytosol-dependent rad-GTPase activity (Fig. 7B). Antibody III inhibited rad-GTPase activity to a lesser extent (36% inhibition) than antibody I (Fig. 7B).

DISCUSSION

The aim of this work was to characterize the endogenous gene product for rad from muscle. We report for the first time several findings, which include showing 1) rad was a 46 kDa protein expressed predominantly in skeletal muscle and heart and its expression increased during myotube formation, 2) endogenous rad protein and mRNA were not overexpressed in muscle from normal, insulin-resistant, or diabetic Pima Indians and the ZDF rat, 3) endogenous rad localized to thin filaments in skeletal muscle, 4) amino acids (109–121), (178–195) and (254–271) may be exposed epitopes on the surface of the native protein, 5) reduced sulfhydryls may be needed for efficient ligand binding, and 6) amino acids (109–121) may be an important structural domain regulating GTPase activity.

Previous studies indicate that expression of rad mRNA is elevated eight- to tenfold in muscle from type II (NIDDM) diabetics compared to either type I (IDDM) diabetics or nondiabetics [Reynet and Kahn, 1993]. In contrast, we found no substantial change in rad expression in skeletal muscle from diabetics compared to healthy controls, suggesting that overexpression of rad is not an underlying cause of diabetes in Pima Indians or ZDF rats. There are several possible explanations for the differences observed in these two studies. For instance, there may be genetic differences in rad expression in muscle from the Pima Indians used in this study compared to Caucasians used in the previous study [Reynet and Kahn, 1993]. Alternatively, the methods used for patient sampling may contribute to the differences. For example, in this study we used muscle biopsies, whereas the other study [Reynet and Kahn, 1993] used amputated leg muscle. Differences in age and sex may also contribute to the differences in both studies. Nevertheless, our results demonstrate expression of rad in Pima Indians and ZDF rats does not correlate with diabetes.

The identification of rad as a protein with a molecular weight of 46 kDa shows it is larger than previously reported for the recombinant form of rad (39 kDa) [Zhu et al., 1995]. One possible explanation for these differences could involve the addition of posttranslational modifications to the endogenous protein which may be absent from the recombinant protein (e.g., phosphorylation, isoprenylation, etc.). However, preliminary experiments revealed no evidence for posttranslational lipid modifications occurring on rad (i.e., myristolation and isoprenylation) [Paulik and Lenhard, unpublished observations]. Another possibility is rad may have an upstream translational start site (i.e., codon 50) different from the one previously suggested (i.e., codon 88) [Reynet and Kahn, 1993]. Consistent with this possibility, GEM (GTP-binding protein induced by mitogens), a homologous member of the rad subfamily, uses the upstream ATG start site rather than the internal site [Maguire et al., 1994]. In any event, the antibodies used in this study demonstrate that endogenous rad migrates as a larger species than the recombinant form of the protein.

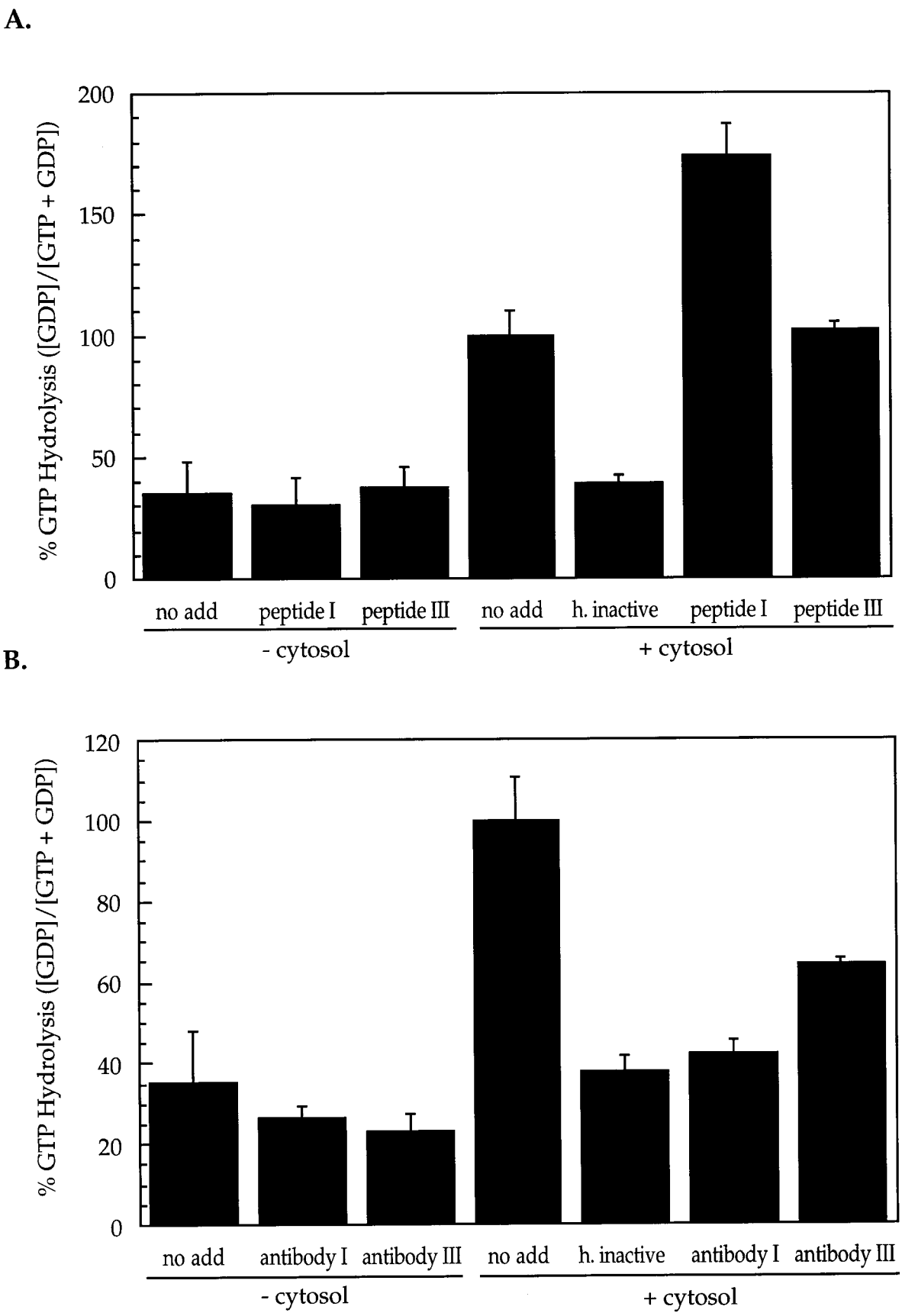

Fig. 7. Characterization of rad GTPase activity. GTPase activity of rad was determined after immobilizing with antibody II on microtiter plates. The GTP-hydrolysis assay consists of loading rad with [α^{32}P]GTP, washing away the free nucleotide, and allowing hydrolysis to occur for 30 min in the presence of 10 mM Mg^{2+} at 25°C. The reaction was stopped, bound nucleotide was eluted, and the sample was analyzed by thin layer chromatography. **A:** Rad GTP-hydrolysis assays were performed in the presence and absence of cytosol and rad peptides I and III or heat (h.) inactivated cytosol. **B:** GTP hydrolysis by rad was determined in the presence or absence of rad antibodies I and III. All data are in triplicate and expressed as a % of the total GTP hydrolyzed.

We made the unique observations that endogenous rad interacts with thin filaments and may be developmentally regulated in skeletal muscle since its expression increases during myotube formation. These observations suggest that rad may be essential in regulating muscle development and function. In a related fashion, the rho family of GTP-binding proteins play an essential role in regulating cell growth and actin polymerization in muscle [Ridley and Hall, 1992]. Further, there are numerous studies implicating auxiliary proteins (i.e., GAP, GEF, and/or GDI) in the regulation of *ras*-related proteins and their interaction with the cytoskeleton [Hall, 1992]. For example, previous work has shown that expression of rac-1 GAP in fibroblasts produces profound changes in the cytoskeletal organization [Herrera and Shivers, 1994]. Similarly, rad's function and/or association with cytoskeletal elements may be regulated by auxiliary protein(s). One hypothesis is that rad may play a role in regulating cell growth by orchestrating cell-matrix and cytoskeletal rearrangements potentially relating to muscle motor functions and/or cytoskeletal architecture. A closer analysis of the functional regulation of rad and its interaction with the cytoskeleton may provide further insight into rad's intracellular role.

There are several possible explanations for the observation that peptide I stimulated rad GTPase activity. First, peptide I may be an agonist for a rad GAP which, in turn, could stimulate rad GTPase activity. Similarly, peptide I may stimulate a GEF which, in turn, would catalyze the replacement of GDP with GTP on rad. Another possibility is that peptide I may antagonize a GDI, thus promoting GDP dissociation from rad. Further, one cannot exclude the possibility that peptide I may affect multiple auxiliary proteins which regulate the GTPase activity of rad. In support for the potential involvement of multiple auxiliary proteins on rad's GTPase activity, previous studies [McKiernan et al., 1993] have shown that rab 3A (*ras*-related GTPases involved in intracellular transport) interacts with three different factors (GAP, GEF, and p85) through the same effector region in the protein.

There are two possibilities which may explain the inhibition of rad-GTPase activity by antibodies I and III. First, these antibodies may inhibit GTP hydrolysis by altering conformational changes within rad. In support of this possibility, the neutralizing antibody to *ras* (Y13-259) alters conformational changes and inhibits GTP-binding, GTPase activity, and autokinase activity of *ras* [Sigal et al., 1986]. Alternatively, antibodies I and III may block the interaction of rad with an auxiliary cytosolic factor(s), such as a GAP and/or a GEF. This possibility is consistent with the observed stimulatory effect of peptide I on rad's GTPase activity.

Taken together, our results suggest that rad and its auxiliary factors may be involved in regulating the cytoskeleton in muscle and that this regulation is not an integral part in the pathogenesis of NIDDM. Moreover, our novel findings on rad's structure and function provides a framework for future studies of rad. These studies should include site-directed mutagenesis of the amino acids (109–121), elucidation of rad's X-ray crystal structure, and the purification and cloning of rad's auxiliary proteins.

ACKNOWLEDGMENTS

We thank Dr. Kathleen Kelly of the NIH for her generous gift of the gem constructs. We are also indebted to the members of the Gila River Indian Community for their long-standing support and participation in our research studies. This work was supported in part by National Institute of Health grants DK 38765 and DK 47461 (to W.T.G) and a merit review grant from the Veterans Affairs Research Committee (to W.T.G).

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