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Human Regulatory Subunit RIβ of cAMP-Dependent Protein Kinases: Expression, Holoenzyme Formation and Microinjection into Living Cells

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The human regulatory subunit RIβ of cAMP-dependent protein kinases was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase. Purification was performed by affinity chromatography on glutathione–agarose beads after cleavage with thrombin. The human recombinant RIβ protein migrated at 55 kDa on SDS–PAGE and displayed immunoreactivity with an anti-human RIβ antiserum. Furthermore, the purified recombinant RIβ protein was shown to exist as a dimer that was able to form holoenzyme with the catalytic subunit Cα. The rate of $RI\beta_2C\alpha_2$ holoenzyme formation was faster in the presence than in the absence of MgATP. The kinase activity measured before and after adding cAMP to the holoenzyme showed that the presence of cAMP resulted in holoenzyme dissociation and release of active Cα-subunit, due to cAMP binding to RIβ. Compared to a $RI\alpha_2C\alpha_2$ holoenzyme, the $RI\beta_2C\alpha_2$ holoenzyme exhibited a more than twofold higher sensitivity to cAMP. The subcellular localization of RIβ was analyzed in quiescent REF-52 fibroblasts and Wistar rat thyroid (WRT) cells after microinjection of fluorescently labeled proteins into the cytoplasm. A cytoplasmic distribution was observed when free RIβ was injected, whereas free Cα injected into the cytoplasm appeared in the nucleus. When holoenzymes with labeled RIβ and unlabeled Cα, or unlabeled RIβ and labeled Cα, were injected, unstimulated cells showed fluorescence in the cytoplasm of both cell types. REF-52 cells stimulated with 8-bromo-cAMP (8-Br-cAMP) and WRT cells treated with thyrotropin (TSH) showed fluorescence mainly in the cytoplasm when RIβ was the labeled subunit of the *in vivo* dissociated holoenzyme. In contrast, nuclear fluorescence was evident from the release and translocation of labeled Cα from the holoenzyme complex after stimulation with 8-Br-cAMP or TSH. © 1994 Academic Press, Inc.

INTRODUCTION

The cAMP-dependent protein kinase (cAK; EC 2.7.1.37)[2] was initially described by Walsh *et al.* [1] and has been shown to be a ubiquitously expressed protein kinase [reviewed in 2–4]. cAK is the key enzyme in the signaling pathway of the second messenger cyclic adenosine 3′,5′-monophosphate (cAMP) and serves as a prototype for the entire family of serine/threonine protein kinases [5]. cAK has, through phosphorylation of target proteins, been implicated in the regulation of metabolism [6, 7], ion transport [8], and gene transcription [9, 10]. The cAK holoenzyme is composed of two regulatory (R) and two catalytic (C) subunits and dissociates upon binding of cAMP to the R-subunits. The R-subunits contain an amino-terminal domain responsible for dimerization, followed by the hinge region containing an autoinhibitor site involved in binding to the C-subunit, and a carboxy-terminal domain with two tandem binding sites for cAMP [for review see 2–4]. Originally, two different R-subunits, type I and type II (RI and RII), were described based on elution from DEAE-cellulose columns [11]. A much greater heterogeneity of cAK subunits is seen at the gene level. The human cAK subunits characterized so far are RIα [12], RIβ [13], RIIα [14], RIIβ [15], Cα [16, 17], Cβ [17], and Cγ [17].

The intracellular distribution of the cAK subunits may provide a mechanism for controlling cAMP-mediated regulation of gene expression, which is presumed to be induced by the catalytic subunit within the nucleus. The RII subunits are associated with the plasma membrane, cytoskeletal components, secretory granules, or the nuclear membrane, through protein–protein interactions between the RII dimer and specific RII-anchoring proteins [reviewed in 18]. These RII-anchoring

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[2] Abbreviations used: C, catalytic subunit of cAK; Cα, Cβ, Cγ, isoforms of C; cAK, cAMP-dependent protein kinases; DTE, dithioerythritol; *E. coli, Escherichia coli;* GST, glutathione *S*-transferase; R, regulatory subunit of cAK; RIα, RIβ, RIIα, RIIβ, isoforms of R; SAC, *Staphylococcus aureus* cells; TSH, thyrotropin.

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proteins are themselves cAK substrates that become phosphorylated at multiple sites [18]. Of the RI subunits, RIα has recently been shown to redistribute to and colocalize with the T-cell receptor complex during activation and capping of human T lymphocytes [19].

The C-subunit can be fluorescently labeled under conditions where nearly full initial phosphotransferase activity is retained [20]. Direct microinjection of fluorescently labeled Cα subunit (or holoenzyme) has confirmed earlier reports of translocation of the C-subunit from the cytoplasm to the nucleus upon cAMP-induced dissociation of the holoenzyme [reviewed in 21]. Microinjected C-subunit is functional in living cells with respect to activation of gene expression [22] and alteration of cytoskeletal structures [23]. Microinjected fluorescently labeled free RIα subunit and RIα_2Cα_2 holoenzyme localize to the cytoplasm. Upon dissociation of the holoenzyme with 8-bromo-cAMP (8-Br-cAMP), free catalytic subunit appears in the nucleus, whereas free RIα remains in the cytoplasm. Microinjection of free catalytic subunit reveals that a major portion of C-subunit is found in the nucleus in the absence of elevated intracellular cAMP [20, 24, 25].

Recent studies from our group and others have indicated subunit-specific effects of the cAK subunits. Such results could provide partial explanations for the multiplicity of cAMP effects seen in cells. These observations necessitate careful studies of each cAK subunit with regard to its biochemical properties, interaction with other proteins, subcellular localization, and functional properties.

In this study, we have expressed the human RIβ subunit in a heterologous system and analyzed its specific biochemical and functional properties in comparison to RIα in holoenzyme complexes with Cα. We also examined the subcellular localization of both the free RIβ and the RIβ_2Cα_2 holoenzyme complex after microinjection of fluorescently labeled proteins into living cells.

MATERIALS AND METHODS

Polymerase chain reaction (PCR). A full-length human RIβ coding region was synthesized by PCR [26] using a 2.4-kb *Eco*RI fragment of the human RIβ cDNA [13] as a template. The upper primer (5'-GCCATGGCCTCCCCGCCCG-3') added the initiation codon ATG to the 5' end of the cDNA and introduced an *Nco*I restriction site. The lower primer (5'-CCAGATCTGCTCAGACGGTGAG-3') introduced a *Bgl*II site directly after the TGA stop codon (both primers were synthesized at Operon Technologies, Alameda, CA). A *Nco*I/*Bgl*II digested PCR fragment was subcloned into the *Nco*I/*Bam*HI sites of the expression vector pET11d (Novagen, Madison, WI). A full-length RIβ protein was expressed at low levels in this expression vector transformed in the *Escherichia coli* strain BL21/DE3 as described elsewhere [27] and indicated that the construct was functional. This full-length protein was used to estimate the correct mobility of the human RIβ on SDS–PAGE (see below).

Expression of fusion protein. To increase the level of expression, the full-length human RIβ cDNA in pET11d was digested with *Nco*I and *Hin*dIII and subcloned into the expression vector pGEX-KG (a kind gift from Dr. J. E. Dixon, Department of Biological Chemistry, University of Michigan, Ann Harbor, MI), containing the glutathione S-transferase (GST) gene. The resulting plasmid was transformed into BL21/DE3 cells, and transformants were screened for expression of fusion protein by analysis on SDS–PAGE as previously described [28]. The expression of fusion protein for purification was performed as follows: 1 liter 2× YT medium containing 20 μg/ml ampicillin was inoculated with 10 ml overnight culture, shaken vigorously at 37°C until OD_{600} reached 0.4–0.5, before adding IPTG to a final concentration of 0.4 m*M*, and incubation continued for 3–4 h. All subsequent procedures were carried out at 4°C. Cells were harvested by centrifugation at 5000 rpm for 5 min, and the pellet was resuspended in 10 ml phosphate-buffered saline buffer (PBS; 150 m*M* NaCl/20 m*M* sodium phosphate, pH 7.3) containing 2 m*M* EDTA, 0.1% β-mercaptoethanol, 0.2 m*M* phenylmethylsulfonyl fluoride, 5 m*M* benzamidine, and 1% Triton X-100. Subsequently, 2 mg/ml lysozyme, 40 m*M* $MgCl_2$, and 10 μg/ml DNase were added, and the cells were sonicated 3 × 45 s in a Fisher sonic dismembrator Model 300 and centrifuged at 10,000 rpm for 10 min. The supernatant containing soluble proteins was used for purification.

Purification and cleavage of fusion protein. Two milliliters of glutathione–agarose beads (Sigma; 75 mg = 1 ml gel) was preswollen in PBS on a rotating platform at 4°C for 30 min and washed three times in PBS. The beads were added to 10 ml fusion protein supernatant (see above) and incubated for 1 h with rotation. The mixture was packed on a column and washed in PBS until the OD_{280} of the eluate equaled the OD_{280} of the washing buffer. Fusion protein was eluted by competition with free glutathione using 10 ml of 50 m*M* Tris–HCl (pH 8.0) containing 5 m*M* glutathione. The OD_{280} was determined in the eluted fractions, and free glutathione was removed by dialyzing overnight against thrombin cleavage buffer (50 m*M* Tris–HCl, pH 8.0, 150 m*M* NaCl, 2.5 m*M* $CaCl_2$, and 0.1% β-mercaptoethanol). Approximately 2 mg fusion protein was incubated for 1 h on ice in 2 ml thrombin cleavage buffer with 4 μg thrombin. Two milliliters glutathione–agarose beads containing 0.05% Triton X-100 was added and incubation continued at 4°C overnight on a rotating platform. Centrifugation at 5000 rpm for 10 min recovered the supernatant containing the purified human RIβ protein. Purified protein was dialyzed against storage buffer: 20 m*M* potassium phosphate (pH 6.4–6.6), 2 m*M* EDTA, 5 m*M* β-mercaptoethanol, and 20% glycerol. The molar amount of RIβ present was calculated by determining the [^{3}H]cAMP binding activity assuming two cAMP-binding sites per monomer.

Amino acid sequencing. In order to verify the amino acid sequence of the expressed RIβ protein, the amino-terminal sequence of the protein as well as of the degradation products were determined. The protein was incubated overnight at room temperature, subjected to SDS–PAGE, and electroblotted onto PVDF membranes (Bio-Rad Trans-Blot). The membranes were Commassie blue stained and bands of interest were excised and sequenced [29]. The amino acid sequencing was performed on a gas phase sequencer with an on-line PTH analyzer (Applied Biosystems, Model 470A).

DNA sequencing. Nucleotides 141–440 of the previously cloned human RIβ cDNA [13] were sequenced by Lark Sequencing Technologies Inc. (Houston, TX). Standard dideoxy chain termination technique [30] was employed. Severe GC band compressions were resolved using 7-deaza dITP. The sequencing reactions were analyzed on 6% polyacrylamide wedge gels containing 8 *M* urea.

8-Azido-[^{32}P]cAMP photoaffinity labeling and SDS–PAGE mobility. Purified human R-subunits (1 ng/μl), RIβ expressed in vector pET11d, RIβ expressed in vector pGEX-KG, testis RIα [31], and testis RII (RIIα, RIIβ) [31], were incubated in the dark with 1 μ*M* 8-azido-[^{32}P]cAMP (ICN, Irvine, CA) in 50 m*M* Tris–HCl (pH 7.4) for 1 h at 4°C. Covalent incorporation was accomplished by exposure of the reactions to uv light (254 nm) at a distance of 5 cm for 15 min at 20°C. Subsequently, samples were boiled in SDS–PAGE sample buffer and separated on 7.5% slab gels [32]. Gels were subjected to

autoradiography employing Hyperfilm MP (Amersham, Buckinghamshire, UK) and Super Rapid screens (Kodak, Eastman, Rochester, NY).

Immunoprecipitation of photoaffinity-labeled human RIβ. The recombinant human RIβ was photoaffinity labeled as described above, using different amounts of protein (2.5, 10, and 40 ng), and subsequent immunoprecipitation was performed using an anti-human RIβ antiserum (anti-RIβ; 1/15 dilution), raised against a synthetic peptide corresponding to amino acids 61–79 of the human RIβ [32]. As controls, 40 ng of labeled RIβ was incubated with preserum (pre-anti-RIβ; serum prior to immunization from the rabbit used to generate the anti-human RIβ antiserum) and with a monoclonal antibody against human RIα (anti-RIα; 1/50 dilution)[31], respectively. The immunoprecipitations were performed as described elsewhere [32]. Shortly, *Staphylococcus aureus* cells (SAC) expressing protein A were added, and the antigen–antibody complexes bound to protein A were pelleted by centrifugation. The SAC pellets were washed, subsequently boiled in SDS-sample buffer, and subjected to SDS–PAGE together with 5 ng RIβ protein labeled in the absence (−) or presence (+) of excess (100 μ*M*) unlabeled cAMP. The gel was subjected to autoradiography.

SDS–PAGE under nonreducing/reducing conditions. To analyze whether the recombinant RIβ protein existed in a monomeric or dimeric state after purification, the protein was subjected to nonreducing/reducing SDS–PAGE. Approximately 200 ng protein was incubated in SDS-loading buffer (62.5 m*M* Tris–HCl, pH 6.8, 2.3% SDS, 10% glycerol, and 0.001% bromophenol blue) for 1 h with or without 5% β-mercaptoethanol, before heating to 60°C and loading onto an 12.5% SDS–PAGE gel. All of the SDS–PAGE gels were stained in Commassie brilliant blue R-250 and fixed before drying.

Sucrose gradients. One microgram of RIβ protein was photoaffinity labeled as described above and incubated for 30 min in the absence or presence of 1 m*M* dithioerythritol (DTE) and 1 *M* NaCl. Subsequently, samples (300 μl) were subjected to ultracentifugation in sucrose gradients, using BSA as reference standard. The gradients (5–20%, w/v) were prepared in 10 m*M* potassium phosphate (pH 7.4), 1 m*M* EDTA in the absence or presence of 1 m*M* DTE, and 1 *M* NaCl, using a Buchler gradient apparatus. Centrifugation (120,000*g*, 16 h) was performed at +4°C in a SW55Ti rotor and gradients were then fractionated from the bottom. Each fraction (130 μl) was counted in 3 ml scintillation fluid (Optifluor, Packard). The sedimentation of RIβ is plotted in the absence and presence of reducing agent and salt. The sedimentation of BSA in both gradients is indicated as reference.

Time-dependent holoenzyme formation and activation. Reconstitution of holoenzyme was performed with purified recombinant proteins. The human RIβ (see above) and the bovine RIα [33] were separately combined with mouse Cα [34]. The holoenzymes were formed by mixing C-subunit with a 1.5-fold excess of R-subunit at 20°C and dialyzing against a buffer containing 25 m*M* potassium phosphate (pH 6.6), 5% glycerol, 5 m*M* β-mercaptoethanol, in the absence or presence of 1 m*M* $MgCl_2$/100 μ*M* ATP. Aliquots were removed at various time intervals and holoenzyme formation was determined as the percentage of kinase activity in the absence versus the presence of excess cAMP using the coupled spectrophotometric method [35]. The dissociation of the holoenzyme complexes as a function of cAMP concentration was determined by the same method, and the results were calculated as the ratio of catalytic activity in the sample compared to the activity at saturating amounts (10 μ*M*) of cAMP. All assays were carried out in duplicate.

Preparation of proteins for microinjection. The mouse Cα subunit was processed for microinjection mainly as described previously [20, 25]. Briefly, 1–2 mg of protein was exchanged into labeling buffer (100 m*M* Hepes, pH 8.0/8 m*M* $MgCl_2$) by chromatography through a Sephadex G-25 column (5 ml, Pharmacia, Sweden). After adding 5 mM ATP to protect the active site, the protein was labeled on lysine residues by adding a 35-fold molar excess fluoroisothiocyanate (FITC, isomer I). After 30 min at room temperature, the reaction was quenched by adding 100 μ*M* β-mercaptoethanol. The Sephadex G-25 column was equilibrated in injection buffer (5 m*M* sodium phosphate, pH 7.4/100 m*M* KCl), and the labeled protein was separated from free dye and exchanged into injection buffer on the column. The human RIβ subunit was labeled either on lysine residues with 25-fold molar excess tetramethylrhodamine isothiocyanate (TRITC, isomer G) in 25 m*M* Bicine, pH 8.0/0.1 mM EDTA [24, 36] or on cysteine residues with 60-fold molar excess 5-iodoacetamido-fluorescein (5-IAF) in 100 m*M* Hepes, pH 8.0/0.1 m*M* EDTA. The reactions were kept on ice overnight before separation of labeled protein from free dye and exchanging into injection buffer as described above for the C-subunit. Protein concentrations were determined by measuring OD_{280} followed by correction for the contribution of fluorescent dye. Covalent attachment of fluorochromes to the proteins was verified by SDS–PAGE and visualization under uv light followed by Commassie staining. The same procedure ascertained that no major proteolysis took place. Holoenzyme formation was performed in the presence of MgATP as described above, using FITC-labeled Cα and a 1.2-fold excess of unlabeled RIβ or TRITC-labeled RIβ and a 1.2-fold excess of unlabeled Cα. Holoenzyme formation and subsequent dissociation by cAMP was confirmed by the spectrophotometric kinase assay and exchanged into injection buffer as described above. Labeled holoenzymes, free 5-IAF-labeled RIβ and FITC-labeled Cα were sterile filtrated through 0.2-μm filters and concentrated on Centricon-30 columns (Amicon, Beverly, MA) to a concentration of about 10 mg/ml.

Cell cultures and microinjections. REF-52 fibroblasts and Wistar rat thyroid (WRT) cells were essentially treated as described previously [20, 37]. The REF-52 cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 200 μg/ml geneticin (G418), whereas the WRT cells were propagated in 6H medium [38] consisting of Coon's modified Ham's F-12 medium supplemented with hydrocortisone, glycyl-L-histidyl-lysine, insulin, transferrin, somatostatin, and thyrotropin (TSH), in addition to 5% calf serum. Both media contained 2 m*M* L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For injection, the cells were plated on scored coverslips, grown to subconfluence, and then incubated in serum-free, hormone-free medium for at least 24 h. Proteins were microinjected into the cell cytoplasm to a final intracellular concentrations of approximately 2.5–5 μ*M*. In both cell types, free 5-IAF-labeled RIβ, free FITC-labeled Cα, holoenzyme with TRITC-labeled RIβ, or FITC-labeled Cα was microinjected into individual cells, using pressure injection and automated micromanipulators (Eppendorf Automatic Micromanipulator). Microinjected REF-52 and WRT cells received no treatment, or the REF-52 cells were stimulated with 1 m*M* 8-Br-cAMP, whereas the WRT cells were incubated with 1 n*M* TSH. One hour after injection, the cells were fixed in 3.7% formaldehyde/PBS for 5 min at 22°C and mounted in PBS containing 15% Gelvatol (polyvinyl alcohol), 33% glycerol, and 0.1% NaN_3. The cells were observed and photographed with a Zeiss Axiophot fluorescence microscope under a 40× or 63× (1.4 numerical aperture) oil-immersion lens. Photographs were made with Kodak T-Max films.

RESULTS

Protein Purification

The GST–RIβ fusion protein was expressed at high levels in *E. coli*, and purified by glutathione–agarose affinity chromatography [39]. Twenty milligrams of protein was purified per liter lysate. The purified fusion protein migrated with an apparent molecular mass of approximately 80–81 kDa following SDS–PAGE (Fig. 1, lane 1). After cleavage from GST using thrombin, the purified recombinant human RIβ protein with an

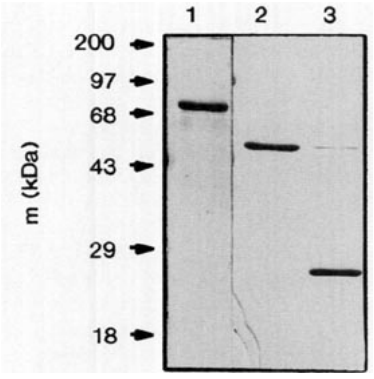

FIG. 1. Purification and thrombin cleavage of fusion protein. Lane 1, fusion protein; lane 2, supernatant after cleavage of fusion protein by thrombin; and lane 3, proteins remaining on the glutathione–agarose beads after cleavage of fusion protein by thrombin. The proteins were subjected to 12.5% SDS–PAGE, and the gel was Commassie stained, fixed, and dried. The approximately molecular mass of the fusion protein was 80–81 kDa, GST was 26 kDa, whereas human RIβ was 55 kDa. Molecular mass (m) in kDa of protein standards (High Molecular Weight, Bio-Rad) are indicated on the left.

amino-terminal extension of 15 amino acids, was shown to give a band on SDS–PAGE of approximately 55 kDa (Fig. 1, lane 2), whereas GST gave a band of 26 kDa (Fig. 1, lane 3).

Sequence of the Hinge Region of Human RIβ

The recombinant RIβ protein was susceptible to limited proteolysis, and degradation products of approximately 40 and 15 kDa were observed on SDS–PAGE after storage at −20°C. Storage at room temperature resulted in further degradation and additional products of approximately 35 and 20 kDa were observed.

Amino acid sequencing revealed the proteolytic fragments of human RIβ to be a mixture of fragments with one cleavage site after Lys-91 and one after Tyr-112. The endogenous cleavage site after Lys-91 lies near the hinge region, and this region is also known to be susceptible to proteolysis in the RIα protein [40-42]. Tyr-112 lies between the consensus inhibitor site and cAMP-binding site A. Amino acid sequencing of the cleavage fragments and subsequent nucleotide sequencing of the previously cloned human RIβ [13] revealed that the published sequence of human RIβ [13] has to be corrected (Fig. 2). The corrected deduced and sequenced amino acid sequence in the hinge region of human RIβ is similar to the published mouse RIβ, -RRRRGGVSA- (Fig. 2B) [43], starting at amino acid 92 [13]. The NH_2-terminus of the recombinant human RIβ was verified to match the deduced amino acid sequence from the published RIβ cDNA [13].

SDS–PAGE Mobility of Human R-subunits

In order to more precisely determine the mobility of the human RIβ, a construct (RIβ-pET11d) was used for expression that yielded a full-length RIβ with no additional amino acids. Expression of this construct and subsequent purification on a cAMP-affinity column resulted in low levels of human RIβ (data not shown). Figure 3A shows the 8-azido-[^{32}P]cAMP labeled purified human RIβ in comparison to the photoaffinity-labeled purified human testis RIα and RII. Human testis RIα has previously been reported to have a molecular mass of 49 kDa on SDS–PAGE [31] (lane 1), whereas human testis RII contains two bands of 8-azido-[^{32}P]cAMP-labeled proteins (lane 3). The lower band represents dephosphorylated RIIα (51 kDa), whereas the upper band represents RIIβ (53 kDa) [31]. Purified full-length human RIβ (lane 2) is slightly larger than RIIβ and has an apparent molecular mass of 54 kDa.

Comparison of the RIβ protein expressed either in the pET11d or in the pGEX-KG system is shown in Fig. 3B. This demonstrates that the 15-amino-acid N-terminal extension of RIβ slightly increases the SDS–PAGE mobility of the RIβ protein. Whereas the full-length protein has a SDS–PAGE mobility of 54 kDa (RIβ, lane 1), the fusion protein appears to migrate as a 55-kDa protein (RIβ*, lane 2).

Antibody Reactivity and Specificity

8-azido-[^{32}P]cAMP photoaffinity-labeled RIβ was incubated in solution with a specific anti-human RIβ antiserum (anti-RIβ) and a monoclonal RIα antibody (anti-RIα), respectively. Figure 4 demonstrates that RIβ was photoaffinity labeled by 8-azido-[^{32}P]cAMP in the absence of excess unlabeled cAMP, whereas the presence of unlabeled cAMP (100 μM) completely abolished the photoaffinity labeling (left lanes, − and +). The anti-RIβ anti-serum precipitated the photoaffinity-labeled protein when 10 and 40 ng of labeled protein were used (middle lanes). No immunoprecipitation of RIβ occurred in the presence of the RIβ preimmune serum (pre-anti-RIβ) and no cross-reactivity was observed between the RIβ protein and the monoclonal antibody against human RIα (anti-RIα, right lanes) [32].

The Purified RIβ Protein Exists as a Dimer

In order to analyze the functional characteristics of the recombinant RIβ protein, we investigated if the recombinant RIβ preparation contained dimeric protein. The expressed protein was subjected to electrophoresis either in the presence or absence of reducing agents (Fig. 5A). The purified RIβ protein migrated as a monomer (55 kDa) in the presence (lane 2) and as a dimer (110 kDa) in the absence (lane 3) of β-mercaptoethanol, respectively. This suggests that the protomers of the dimer are covalently cross-linked by interchain disulfide bonds similarly to the RIα subunit [44].

In addition, the purified protein was sedimented on sucrose gradients under nondissociating conditions

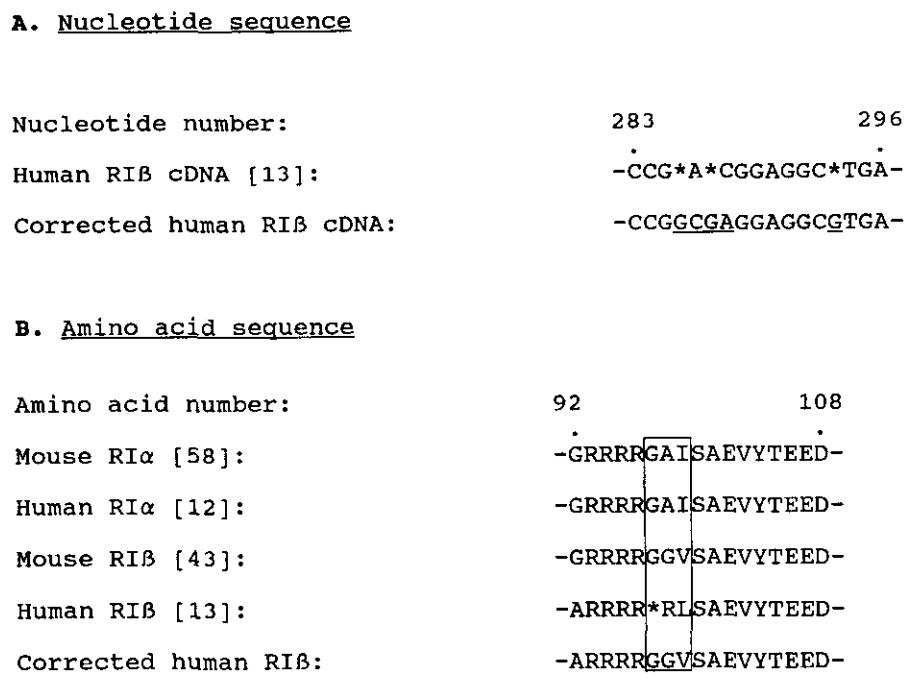

FIG. 2. Corrected nucleotide and amino acid sequence in the hinge region of the human regulatory subunit RIβ of cAMP-dependent protein kinases. The hinge region in mouse and human RIα [12, 58] and mouse RIβ [43] are shown in comparison with the previously published [13] and corrected sequence of the human RIβ (this study). The asterisks, introduced in the previously published human RIβ sequence, represent the nucleotides (A) or the amino acids (B) not earlier detected in this region. The nucleotide and amino acid numbering (above the sequences) correspond to the previously reported human RIβ sequence. The sequence data are available from EMBL/GenBank/ODBJ under Accession No. M65066.

(Fig. 5B, closed squares) versus dissociating conditions (1 m*M* DTE/1 *M* NaCl; Figure 5B, open circles), respectively. The mobility shift of the fractionated protein under dissociating versus nondissociating conditions demonstrates that the expressed purified protein is present in the preparations as a dimer under nondissociating conditions. The same mobility shift was also observed for bovine RIα using the same conditions (data not shown). Furthermore, sucrose gradient centrifugation in the presence of reducing agent and absence of salt revealed that more than 50% of RIβ still sedimented as a dimer (data not shown). This strongly indicates that other noncovalent interactions between the RIβ protomers are also important for dimerization.

Holoenzyme Formation

Reconstitution of holoenzymes was performed by dialyzing a 1.5-fold excess of human RIβ or bovine RIα with mouse Cα in the absence or presence of MgATP (Fig. 6). The holoenzymes were allowed to form for 48 h, and catalytic activity was measured at different time points. RIβ was shown to form holoenzyme with the catalytic subunit Cα, thereby blocking the catalytic activity in the absence of cAMP. Holoenzyme formation with either human RIβ (Fig. 6, circles) or bovine RIα (Fig. 6, squares) proceeded rapidly in the presence of MgATP (Fig. 6, filled symbols), whereas the absence of MgATP (Fig. 6, open symbols) reduced the rate, but not the extent, of holoenzyme formation.

cAMP Activation of Holoenzymes

The cAMP-binding properties were investigated by measuring the cAMP activation of holoenzymes composed by dialyzing overnight either RIβ or RIα with Cα in the presence of MgATP (see above; Fig. 7). Binding of MgATP to the type I holoenzyme has earlier been shown to increase the half-maximal activation, $K_{act(cAMP)}$, of the holoenzyme presumably because the C-subunit/MgATP complex facilitates the dissociation of cAMP from the R-subunit [45]. The RI holoenzymes, in the absence of cAMP, were enzymatically inactive complexes *in vitro*, with neglectable kinase activities. Upon addition of cAMP, the holoenzymes dissociated, and the $RI\beta_2C\alpha_2$ holoenzyme (Fig. 7, circles) was observed to be half-maximally activated at a 2.1-fold lower cAMP concentration compared to the $RI\alpha_2C\alpha_2$ holoenzyme (Fig. 7, squares). The apparent $K_{act(cAMP)}$ for the $RI\beta_2C\alpha_2$ and $RI\alpha_2C\alpha_2$ holoenzymes were 83 and 172 n*M*, respectively. Positive cooperativity was observed for both holoenzymes, with Hill coefficients of 1.7 for the

$RI\beta_2C\alpha_2$ holoenzyme and 2.2 for the $RI\alpha_2C\alpha_2$ holoenzyme, respectively.

Binding of [^{3}H]cAMP to dimeric human RIβ was also measured as a function of cAMP concentration. This experiment showed that the RIβ dimer bound cAMP in a concentration-dependent fashion (cAMP binding constant, $K_{a(cAMP)}$ of 50 nM; data not shown).

To further analyze the stability of the $RI\beta_2C\alpha_2$ holoenzyme, the dissociation constant, $K_{d(holoenzyme)}$, for holoenzyme formed with FITC-labeled Cα-subunit (see above) in the absence and presence of MgATP, respectively, was measured by fast-phase liquid chromatography (FPLC; data not shown). In the absence of MgATP, the apparent $K_{d(holoenzyme)}$ was estimated to be 0.1 μM, whereas the apparent $K_{d(holoenzyme)}$ in the presence of MgATP was lower than 0.13 nM. This method [46] , in addition to the above mentioned holoenzyme activation, shows not only that RIβ is capable of forming a very stable complex with the catalytic subunit but also that this complex is dependent on MgATP for stability, and that dissociation of the complex is mediated by cAMP.

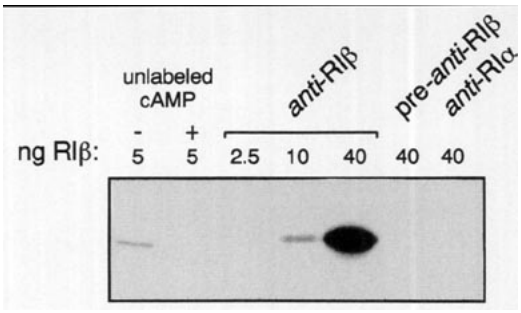

FIG. 4. 8-Azido-[^{32}P]cAMP labeling of human RIβ and immunoprecipitation. The recombinant human RIβ (amounts indicated) was photoaffinity labeled with 8-azido-[^{32}P]cAMP and incubated in solution with an antiserum against RIβ (anti-RIβ). As controls, 40 ng RIβ protein was incubated with preserum (pre-anti-RIβ) or a monoclonal RIα antibody (anti-RIα). The antigen–antibody complexes were precipitated by binding to *Staphylococcus aureus* cells expressing protein A and pelleted by centrifugation. In addition, 5 ng RIβ protein was labeled in the absence (−) or presence (+) of excess (100 μM) unlabeled cAMP. The samples were boiled in SDS-sample buffer, the labeled proteins were separated on SDS–PAGE and subjected to autoradiography.

Free RIβ Subunit and $RI\beta_2C\alpha_2$ Holoenzyme Are Cytoplasmic

Human RIβ and mouse Cα were labeled with fluorochromes (see Materials and Methods). Both the R- and C-subunits retained their functional activity and the ability to reassociate to form holoenzyme, which in turn dissociated upon the addition of cAMP. Initially, cAK holoenzymes with FITC-labeled Cα and TRITC-labeled RIβ were microinjected, since this labeling approach has been the most successful for the $RI\alpha_2C\alpha_2$ holoenzyme [36]. Furthermore, we were able to obtain free RIβ protein labeled with 5-IAF, most probably due to two additional cysteine residues on the amino terminus of human RIβ compared to RIα [13]. Free 5-IAF-labeled RIβ was more stable than the free TRITC-labeled RIβ subunit.

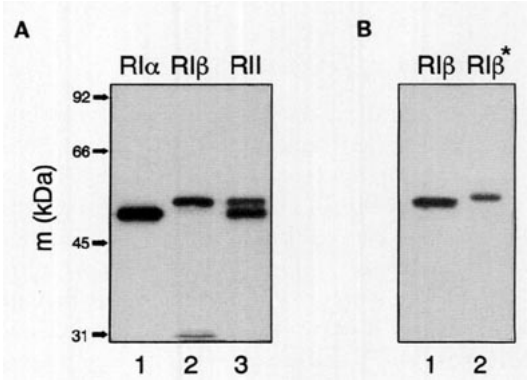

FIG. 3. Comparison of the relative SDS–PAGE mobilities of 8-azido-[^{32}P]cAMP-labeled human R-subunits. (A) Proteins (10 ng) were photoaffinity labeled with 8-azido-[^{32}P]cAMP and subjected to 7.5% SDS–PAGE before autoradiography. Lane 1, purified human testis RIα [31]; lane 2, human recombinant full-length RIβ expressed in the pET11d system; and lane 3, purified human testis RII [31]. Molecular mass (m) in kDa of protein standards (High Molecular Weight, Bio-Rad) are indicated on the left. (B) Comparison of the SDS–PAGE mobility of 8-azido-[^{32}P]cAMP-labeled RIβ protein expressed in the pET11d system (RIβ, lane 1) and in the pGEX-KG system (RIβ*, lane 2). The gels were dried before autoradiography.

Labeled subunits were microinjected into the cytoplasm of serum-starved REF-52 fibroblasts and fixed after 1 h (Fig. 8). After injection of free 5-IAF-labeled RIβ protein, the fluorescence was found predominantly in the cytoplasm (Fig. 8A). Some staining of the nucleus could be seen (Fig. 8A–8C), most probably due to optical contamination from the cytoplasm above or below the nucleus. Free FITC-labeled Cα was transported from the cytoplasm into the nucleus after injection resulting in strong nuclear fluorescence (Fig. 8D) and morphological changes (cell rounding), indicative of catalytic activity *in situ*.

Reconstituted holoenzyme formed with fluorescently labeled RIβ (Figs. 8B and 8C) or Cα (Fig. 8E and 8F) was examined after injection in REF-52 cells. The injected holoenzyme appeared in the cytoplasm of unstimulated cells independent of whether RIβ or Cα was labeled (Figs. 8B and 8E). Upon treatment of the cells with 1 mM 8-Br-cAMP (Figs. 8C and 8F) significant differences in the observed distribution of holoenzyme containing TRITC-labeled RIβ (Fig. 8C) and holoenzyme containing FITC-labeled Cα (Fig. 8F) were seen. After addition of 8-Br-cAMP, a cytoplasmic fluorescence was observed for holoenzyme containing labeled RIβ. In contrast, for cells injected with holoenzyme containing labeled Cα, strong fluorescence was also observed in the nucleus (Fig. 8F). Cytoplasmic staining was still ob-

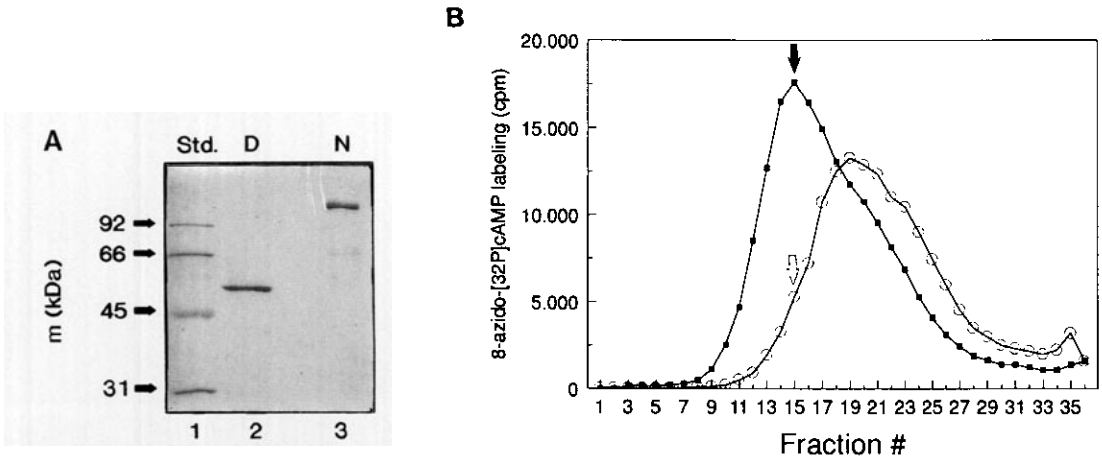

FIG. 5. Separation of RIβ mono- and dimer. (A) SDS–PAGE mobility of RIβ in the absence and presence of reducing agent (5% β-mercaptoethanol). Lane 1, molecular mass (m) in kDa of protein standards (High Molecular Weight, Bio-Rad). Lane 2, RIβ mobility in presence of reducing agent (D); lane 3, RIβ mobility in absence of reducing agent (N). (B) Sucrose gradient centrifugation of 8-azido-[^{32}P]cAMP-labeled RIβ. Nondissociating conditions (■), dissociating conditions (O; 1 m*M* DTE/1 *M* NaCl).

served to some extent, but the main localization was clearly nuclear.

Similarly to the treatment regime for REF-52 cells (Fig. 8), a set of experiments were performed in WRT cells showing an identical distribution of microinjected fluorescently labeled RIβ, Cα, and holoenzymes (Fig. 9). Free 5-IAF-labeled RIβ (Fig. 9A) and TRITC-labeled RIβ holoenzyme (Fig. 9B) were located in the cytoplasm. In cells stimulated with 1 n*M* TSH (Fig. 9C) to dissociate the holoenzyme, the fluorescence remained cytoplasmic. In contrast, free FITC-labeled Cα (Fig. 9D) appeared in the nucleus, although undissociated holoenzyme with labeled Cα was cytoplasmic in untreated cells (Fig. 9E). Upon TSH stimulation (Fig. 9F) the holoenzyme dissociated and the Cα-fluorescence was redistributed to the nucleus. Together, the two regimes demonstrate that free RIβ and holoenzyme do not accumulate in the nucleus, unlike free Cα-subunit, but are mainly cytoplasmic.

DISCUSSION

Although RIβ mRNA mainly appears in nervous tissues in mouse [13, 43, 47–49], and has been detected in rat brain and testis [50], a low-level expression has been

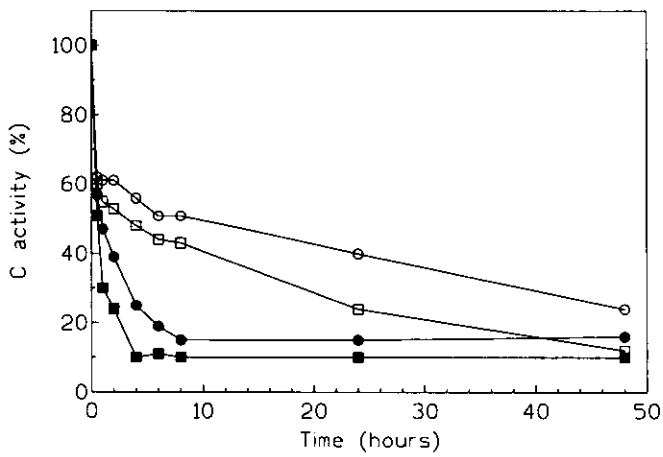

FIG. 6. Holoenzyme formation of human RIβ versus bovine RIα, when combined with mouse Cα in the absence and presence of MgATP. The activity ratio represents activity in the absence of cAMP versus the activity in the presence of cAMP, where 100% represents full activity of free C-subunit before combining with the RI subunits to holoenzymes. Holoenzyme formed in the absence of MgATP (O, □); holoenzyme formed in the presence of MgATP (●, ■); $RI\beta_2C\alpha_2$ holoenzyme (O, ●); $RI\alpha_2C\alpha_2$ holoenzyme (□, ■).

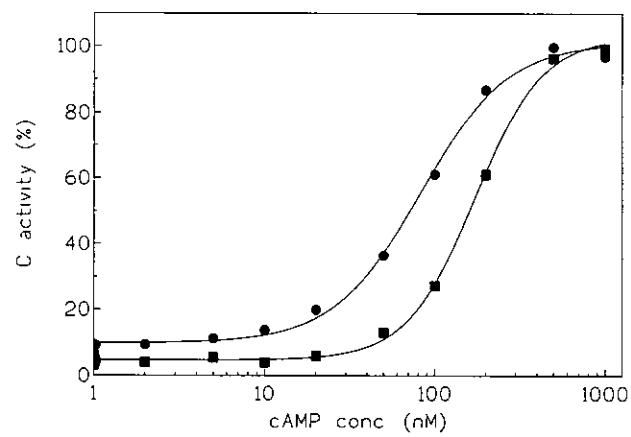

FIG. 7. Activation by cAMP of holoenzymes formed with human RIβ or bovine RIα in the presence of MgATP. A constant amount of holoenzyme was used for all cAMP concentrations (0–1000 n*M*). $RI\beta_2C\alpha_2$ holoenzyme (●); $RI\alpha_2C\alpha_2$ holoenzyme (■). The presented data are representative of three independent experiments.

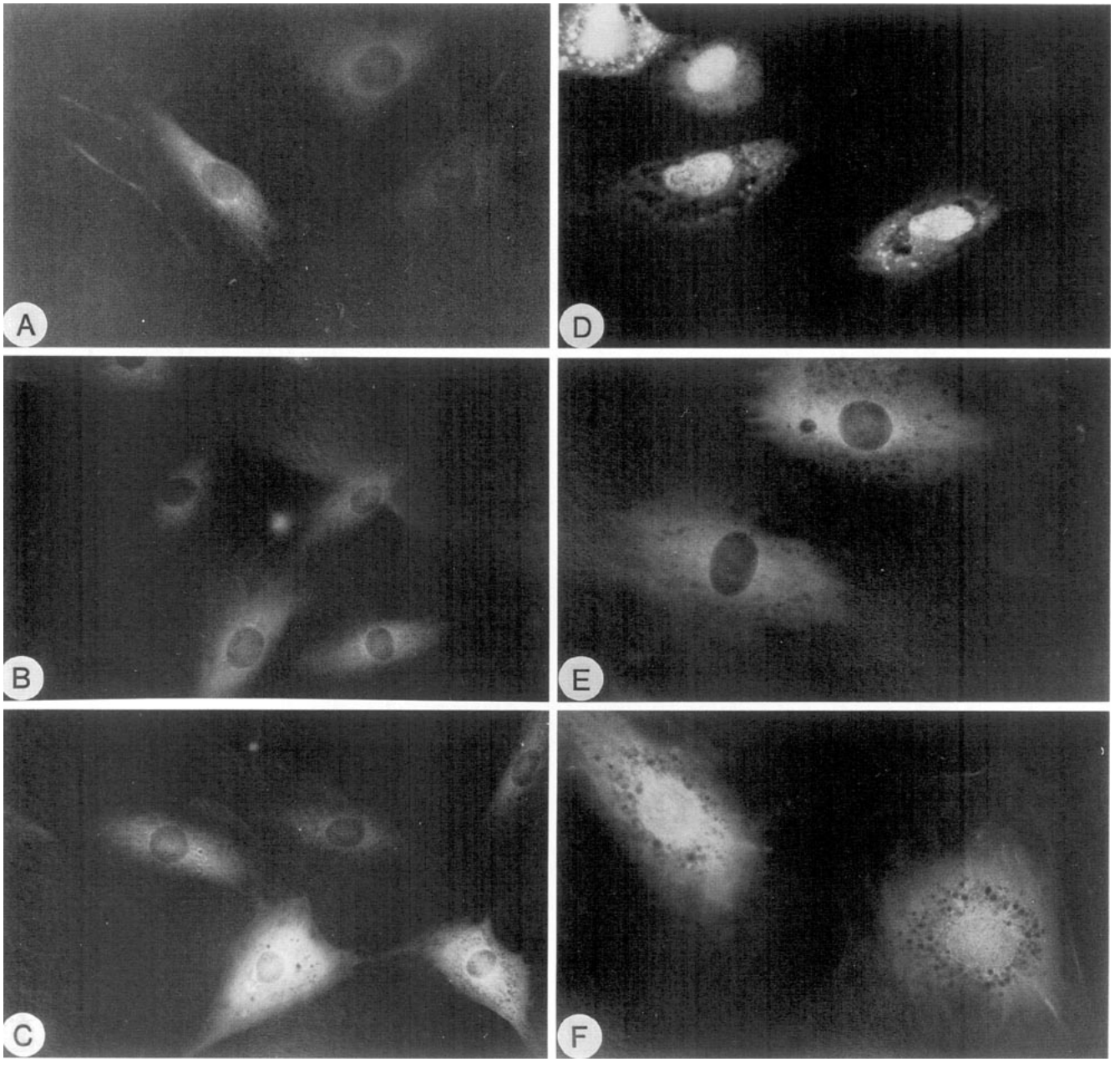

FIG. 8. Fluorescence photomicrographs of REF-52 fibroblasts microinjected with fluorescently labeled proteins into the cytoplasm. (A) Free 5-IAF-labeled human RIβ injected. (B) Holoenzyme with TRITC-labeled RIβ injected in unstimulated cells. (C) As in B, but cells were treated with 8-Br-cAMP. (D) Free FITC-labeled mouse Cα injected. (E) Holoenzyme with FITC-labeled Cα injected in unstimulated cells. (F) As in E, but cells were treated with 8-Br-cAMP. The holoenzymes were formed from purified recombinant human RIβ and mouse Cα. The cells were fixed after 1 h incubation with microinjected proteins.

observed in most human tissues [13]. The functional consequences of this are not known. However, the recent demonstration of RIα–RIβ heterodimer complexes with associated phosphotransferase activity increases the possibilities of diversification of cAMP-mediated effects [32]. Due to these differences among mammalian species, we wanted to investigate further the recombinant human RIβ protein expressed in *E. coli* in order to characterize its biochemical and functional properties.

The present data show a simple and powerful method to isolate and purify human RIβ polypeptide from bacteria. The GST fusion protein expression system adds 15 unrelated amino acid residues at the amino-terminus of the protein (NH_2-GSPGISGGGGGILDS-RIβ), corresponding to the glycine-rich linker after cleavage with

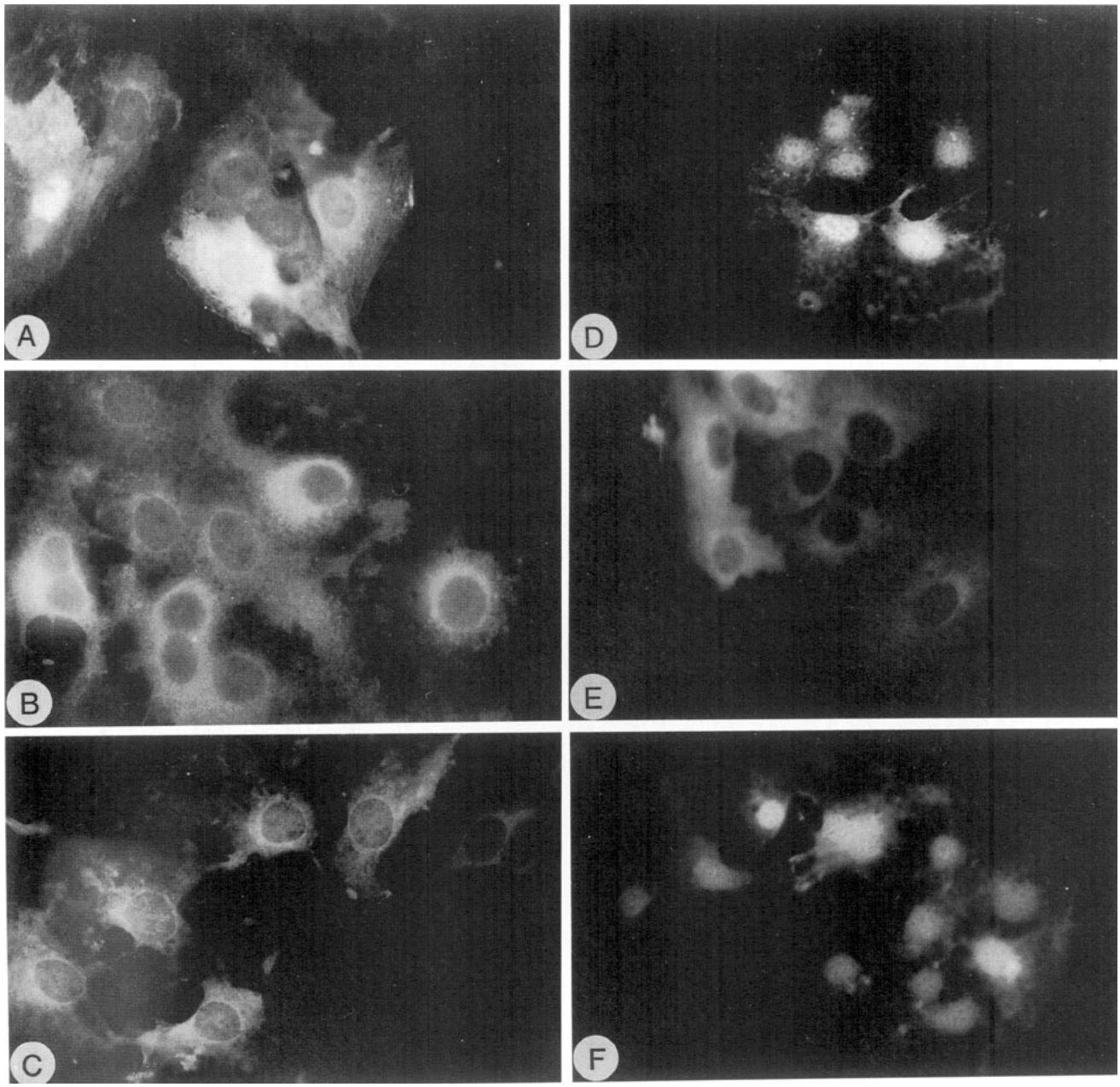

FIG. 9. Fluorescence photomicrographs of Wistar rat thyroid (WRT) cells microinjected with fluorescently labeled proteins into the cytoplasm. (A) Free 5-IAF-labeled human RIβ injected. (B) Holoenzyme with TRITC-labeled RIβ injected in unstimulated cells. (C) As in B, but cells treated with TSH. (D) Free FITC-labeled mouse Cα injected. (E) Holoenzyme with FITC-labeled Cα injected in unstimulated cells. (F) As in E, but cells treated with TSH. The holoenzymes were formed from purified recombinant human RIβ and mouse Cα. The cells were fixed after 1 h incubation with microinjected protein.

thrombin. This amino-terminal extension could influence the structure and activity of RIβ, but the purified human RIβ protein was shown to exist as a dimer and to form holoenzyme with the catalytic subunit. Saraswat *et al.* [33] expressed the bovine RIα subunit as a fusion protein containing 10 additional amino acids of the β-galactosidase gene at the amino-terminus (NH_2-TMITNSPPDS-RIα). This protein appeared to be functioning in an analogous manner to its naturally produced counterpart. Therefore, we assume that the amino-terminal extension on human RIβ does not influence its biochemical or functional properties. Cadd *et al.* [48] expressed the mouse RIβ and demonstrated that the holoenzyme containing mouse RIβ was activated at a 2.5-fold lower concentration of cAMP compared to the holoenzyme containing mouse RIα. In the present

study, we show that human RIβ is 2.1-fold more sensitive to cAMP, compared to bovine RIα, in holoenzyme complexes.

The amino acid residues responsible for the increased cAMP sensitivity of RIβ versus RIα have been examined to some extent. Cadd *et al.* [48] made mutants of the mouse RIβ by converting amino acids of the hinge region which differs between RIα and RIβ. In a single mutant, Gly at position 98 was converted to Ala (RIβ_{Ala}), and in a double mutant Gly and Val at positions 98 and 99 were converted to Ala and Ile (RI$\beta_{Ala/Ile}$). Holoenzymes formed with these mutants gave $K_{act(cAMP)}$ values higher than holoenzyme with wild-type RIβ, but not as high as $K_{act(cAMP)}$ for the holoenzyme with RIα, suggesting that other amino acids as well contribute to the differences in $K_{act(cAMP)}$. Looking at the amino acid similarity between mouse/bovine RIα and mouse/human RIβ, other candidates for this difference in cAMP sensitivity are Ala-110(RIα) → Val-110(RIβ), Ala-125(RIα) → Thr-125(RIβ), and Glu-132(RIα) → Ser-132(RIβ).

The activation constant, $K_{act(cAMP)}$, i.e., free cAMP concentration that gives 50% holoenzyme dissociation and subsequent kinase activity, is about 0.1 μM with positive cooperativity (Hill coefficient 1.2–1.8) for regulatory subunits of cAMP-dependent protein kinases [51-53]. Previous kinetic analysis of cAMP binding indicates that for the free RI subunit, cAMP binds first to site A [54], whereas for the holoenzyme, cAMP binds first to site B [55] possibly due to shielding of site A by the catalytic subunit. Binding of cAMP to site B in the holoenzyme most probably leads to a conformational change that affects the affinity of site A for cAMP [56]. The present data demonstrates the critical role of the presence of MgATP both in the mechanism for reassociation and dissociation of the RIβ subunit and the catalytic subunit. As reported previously, this is due to the high-affinity binding site for MgATP in type I holoenzymes that stabilizes the holoenzyme [57]. It is the catalytic subunit–MgATP complex that is the critical factor for reassociation of the subunits [56], whereas the complex $R_2C_2(MgATP)_2$, once formed, is as well stabilized by MgATP [46].

The kinase activity of C appears to be almost unchanged after labeling with FITC in the presence of MgATP. This allows cAMP-binding to the exogenous holoenzyme, resulting in normal patterns of phosphorylation. Furthermore, TRITC-labeled RIβ forms holoenzyme with C that is activated by cAMP similarly to unlabeled RIβ. In the present study, holoenzyme injected into the cytoplasm remains there and does not seem to enter the nucleus. After cAMP-induced dissociation of the holoenzyme, Cα-subunit translocates to the nucleus, whereas the RIβ subunit remains in the cytoplasm.

In contrast to the anchored RII subunits [18], the RI subunits have been assumed to be soluble and cytoplasmic [20]. Recent data from our laboratory indicates that the regulatory subunit RIα localizes to and interacts with the T-cell receptor complex during activation and capping of human T lymphocytes [19]. Further experiments are in progress to establish the role of cAK type I isozymes during T- and B-cell activation. It would be interesting to establish if RIβ in a similar manner is associated with any particular R-binding protein. One might speculate that specific cell types not investigated (i.e. neurons) have RIβ-binding proteins and that there might be different RIβ-binding proteins in human and rodent cells due to differences in the N-terminal region in human versus rodent RIβ [13]. To address this question, labeling of proteins with fluorescent dye is a powerful tool for visualizing their intracellular distribution and identifying binding to specific cell components or different organelles. By using separately labeled cAK subunits in microinjection studies it is possible to independently track their position in the cell and to follow translocation to other subcellular compartments.

Our data on microinjection of fluorescently labeled proteins shows that free RIβ and reconstituted $RI\beta_2C\alpha_2$ holoenzyme are mainly cytoplasmic, whereas free catalytic subunit is transported from the injection site in the cytoplasm to the nucleus. This data are in agreement with previous data for RIα, Cα, and $RI\alpha_2C\alpha_2$ holoenzyme [20]. Furthermore, the present study reports a simple and rapid method for production of a functional dimeric human RIβ protein that, in a holoenzyme complex with Cα, has a higher affinity for cAMP than RIα. The microinjected RIβ appears to distribute as soluble free RIβ dimer or $RI\beta_2C\alpha_2$ holoenzyme in the cytoplasm of REF-52 fibroblasts and WRT cells. In addition, RIβ acts like an inhibitor by inactivating the catalytic activity and preventing the nuclear translocation of the Cα-subunit, in the absence of elevated cAMP.

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