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A crystallizable form of RIIβ regulatory domain obtained by limited proteolysis

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The type RIIβ regulatory subunit of protein kinase A is primarily expressed in adipose tissue and brain. Knockout mice suggest a role for RIIβ in regulating energy balance and adipose-tissue content, thus making it a potential target for therapeutic intervention in obesity. A truncated version of the RIα subunit has been used in a crystallographic study and was used here to design an analogous RIIβ construct. Despite substantial screening, conditions were not found for the crystallization of the truncated RIIβ subunit. However, limited proteolysis of the full-length RIIβ subunit identified boundaries of the 'hinge' region and a fragment containing the two cAMP-binding domains which did crystallize. A recombinant version of the fragment was expressed and crystallized for X-ray diffraction studies. The crystals belong to the orthorhombic space group *C*222, with unit-cell parameters $a = 91.6$, $b = 105.9$, $c = 85.8$ Å, and diffracted to at least 2.3 Å.

1. Introduction

cAMP-dependent protein kinase (PKA), a major mediator of cAMP signaling, is a heterotetramer consisting of two catalytic (C) monomers and a cAMP-binding regulatory dimer (R). Binding of cAMP to the inhibitory R subunits results in dissociation of the two C subunits with resulting activation of the kinase. Two types of R subunits, RI and RII, have been identified and each has two isoforms, α and β (reviewed in Taylor *et al.*, 1990). The type I and type II enzymes and isoforms are expressed differentially in different tissues. The RIIβ isoform is the major form found in adipose tissue; knockout mice suggest a role for the RIIβ holoenzyme in regulating energy balance and adipose-tissue content (Cummings *et al.*, 1996). Each R subunit contains an N-terminal domain which includes the region required for dimerization and a proteolytically sensitive 'hinge' region which contains an RRXS/A sequence required for interaction with the catalytic subunits. The C-terminal two-thirds of the polypeptide contains two tandem cAMP-binding domains, A and B. The structure of a deletion mutant missing the N-terminal 91 amino acids of the RIα-type subunit has been solved by X-ray crystallography (Su *et al.*, 1995). Although monomeric, it retains the two cAMP-binding sites and therefore should represent a valid surrogate for structural studies aimed at identifying modulators of this kinase regulatory subunit.

In order to grow large single well ordered crystals for X-ray diffraction studies, the protein used must be highly pure, homogeneous and soluble. In addition, multi-domain proteins with highly flexible or membrane-spanning regions are often very difficult to crystallize. In order to overcome this issue, it has become common to identify functional structural domains of such proteins for crystallization. Systematic experiments using intact proteins and small amounts of exogenously added proteases, termed limited proteolysis (reviewed in Hubbard, 1998), can lead to the identification of crystallizable structural domains for X-ray diffraction studies. Once identified, these domains can be purified and used directly or can be expressed in recombinant systems to obtain the multi-milligram amounts needed for crystallographic studies. Here, we report on the design and production of a crystallizable form of the RIIβ regulatory subunit of cAMP-dependent protein kinase using limited proteolysis.

2. Materials and methods

2.1. Expression in *Escherichia coli*

The RIIβ full-length construct was produced by PCR amplification and ligated into pET14b (Novagen). The nucleotide sequence was confirmed. Expression using the T7 promoter system was in LB with ampicillin (100 μg ml^{-1}) at 310 K. Cells were harvested 3 h after induction with 500 μM IPTG.

The RIIβ A/B domain was obtained by PCR from full-length RIIβ and subcloned into pET28a(+) (Novagen). The sequence was confirmed. The construct was expressed in LB media with 50 μg ml^{-1} kanamycin at 301 K.

The cells were grown overnight and harvested (no induction).

The RIIβ Δ108 was PCR amplified from full-length RIIβ, subcloned into pET14 and expressed in *E. coli* strain BL21(DE3) and grown as described for the A/B domain.

2.2. RIIβ purification

Full-length His-tagged RIIβ was purified by Ni–NTA chromatography (Qiagen) and stored at 193 K in 50 m*M* Tris–HCl pH 7, 150 m*M* NaCl, 1 m*M* EDTA, 5 m*M* dithiothreitol (DTT) and PIC (2 m*M* PMSF, 1 μg ml^{-1} each of aprotinin, leupeptin and pepstatin). The His tag was removed by thrombin cleavage and full-length RIIβ purified by chromatography on Mono Q (Pharmacia-LKB).

The RIIβ Δ108 and A/B domains were isolated by Ni–NTA agarose chromatography. The His tag was removed by thrombin cleavage and rechromatography on Ni–NTA. The untagged proteins were then further purified by chromatography on Q Sepharose and Superdex 75. The purified protein was estimated to be greater than 95% pure by silver-stained SDS–PAGE. The protein was stored at 253 K in 20 m*M* Tris–HCl pH 7, 150 m*M* NaCl, 5 m*M* DTT, 5 m*M* EDTA, PIC and 30% glycerol.

The Δ17Δ7 fragment was purified from AspN digests by chromatography on Mono-Q (Pharmacia-LKB) in 25 m*M* Tris–HCl pH 7.5 and 5 m*M* DTT (buffer *A*). The fragment was eluted with a linear gradient of 0–0.5 *M* NaCl. Fractions containing the Δ17Δ7 fragment were pooled and concentrated prior to crystallization trials.

2.3. Limited proteolysis

The purified full-length RIIβ protein was dialyzed overnight into 20 m*M* Mes/NaOH pH 6, 5 m*M* β-mercaptoethanol. Stock solutions of Asp-N, Glu-C, Lys-C, Arg-C, trypsin, elastase and chymotrypsin (Boehringer) were each prepared according to manufacturers' recommendations. Reaction mixtures with full-length RIIβ and each protease at ratios of 10:1 to 500:1(*w*/*w*) were incubated in appropriate buffer for 30 min to 2 h and the reactions stopped with appropriate inhibitors (PMSF, EDTA, leupeptin). Samples were diluted into SDS buffer, boiled and subjected to SDS–PAGE followed by electroblotting to PVDF and N-terminal sequencing on an Applied Biosystems Model 470 sequencer.

For the scaled-up reaction with Asp-N, a 1/100 ratio of Asp-N to RIIβ was incubated in 20 m*M* Tris–HCl pH 7.5. The reaction mixture was incubated overnight at 277 K. EDTA and PMSF were added to terminate the reaction and the sample was dialyzed into buffer *A* for chromatography on MonoQ resin (Pharmacia-LKB).

2.4. Crystallization

The purified RIIβ Δ17Δ7 or A/B proteins in buffer *A* plus 100 m*M* NaCl were concentrated to 20 mg ml^{-1} in a Centricon 10 microconcentrator (Amicon). Protein concentration was determined with Coomassie Plus reagent using BSA standard curves (Pierce Chemicals, Rockford, IL). When used, cAMP was added at 400 μ*M*. Optimal crystals of the D17D7 fragment were grown at 295 K in hanging drops containing protein at 8–10 mg ml^{-1} in 0.05 *M* cacodylate/NaOH pH 6.5, 3–5% PEG 8000 and 0.1 *M* magnesium acetate. Crystals of the A/B construct were grown in drops with 8–10 mg ml^{-1} protein and 4–8% PEG 4000, 0.1 *M* sodium acetate pH 4.6 and reached a maximum size of about 200 $\times$ 200 $\times$ 100 μm after about a week.

2.5. X-ray data collection

Data were collected on a Rigaku RU-H3R rotating anode with a copper target operated at 50 kV, 100 mA using a MAR 30 cm image-plate detector.

The crystal-to-film distance was 150 mm and 1° oscillations were collected.

The crystals were flash-frozen in a stream of nitrogen at 100 K using an Oxford Cryosystems Cryostream cooler. Data were reduced and scaled using *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997). The crystals diffracted to 2.3 Å with R = 0.061 overall. The space group was *C*222, with unit-cell parameters a = 91.57, b = 105.91, c = 85.80 Å.

3. Results and discussion

Since a truncated form of the RIα regulatory subunit had been previously crystallized and the X-ray structure determined (Su *et al.*, 1993, 1995), our initial goal was to produce an analogous construct of RIIβ based on primary sequence alignment of the two regulatory subunits (which are 38% identical; Fig. 1). This construct for RIIβ (Δ108) consisted of residues 108–381. The purified protein was concentrated and subjected to hanging-drop vapor-diffusion screening against Hampton Crystal Screen I (CSI; Jancarik & Kim, 1991). Screens were run at 281 and 295 K both in the presence and absence of added cAMP and at several different protein concentrations, but no crystals were obtained. Screening of ammonium sulfate concentration and pH also gave no crystals. The published conditions of Su *et al.* (1995) for the RIα deletion mutant also failed to give crystals.

In an attempt to obtain a fragment more amenable to crystallization, we subjected full-length RIIβ to limited proteolysis with several proteases of different specificity. The full-length protein formed the expected dimer as determined by size-exclusion chromatography and ultracentrifugation studies (data not shown). Endoproteinases Glu-C, Asp-N, Arg-C, Lys-C, trypsin, elastase and chymotrypsin were incubated in various ratios with the protein and the resulting digests were analyzed by SDS–PAGE. Several of the proteases yielded fragments in the range 35–40 kDa (Fig. 2). These digests were repeated at optimal ratios and subjected to SDS–PAGE

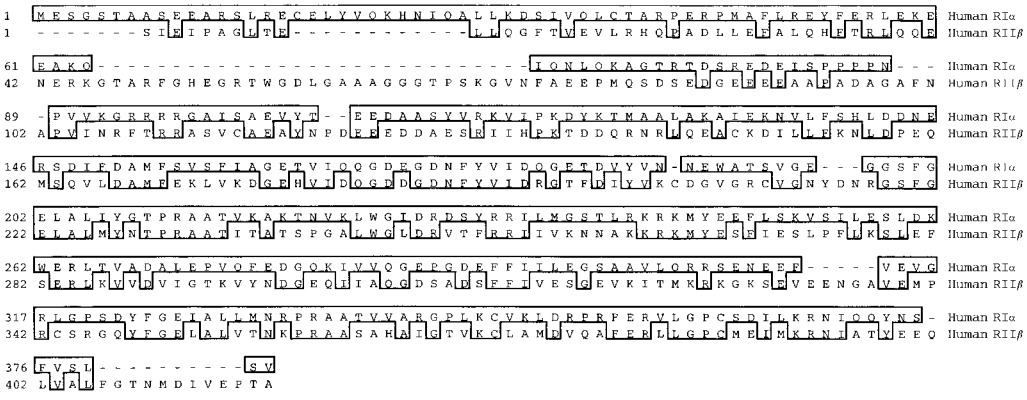

Figure 1
Sequence alignment of human RIα and RIIβ by the Clustal method using the *MEGALIGN* program (DNA Star Inc., Madison, Wisconsin, USA).

followed by electroblotting to PVDF (as described in §2) and to NH_2-terminal sequencing in order to identify the sites of cleavage.

Cleavages were observed in the region of residues 95–128 (Fig. 3). Asp-N cleavage resulted in a fragment of approximately 35 kDa (Fig. 2) with an N-terminal sequence DDAESRI, indicating a fragment with 17 amino acids deleted at the N-terminus relative to the Δ108 construct. This digest of the full length was scaled up and fractionated by chromatography on Mono Q (Pharmacia-LKB). Fractions containing the 35 kDa fragment were pooled, N-terminal sequence verified and then subjected to analysis by LC-coupled electrospray mass spectrometry (LCMS). A mass of 31 940 Da was obtained, consistent with a fragment that in addition to the N-terminal truncation had been truncated at the COOH-terminus by seven amino acids. The fragment, which we designated RIIβΔ17Δ7, was monomeric on SEC and bound cAMP (data not shown).

The purified Δ17Δ7 fragment was concentrated and set up in vapor-diffusion crystallization trials using the Hampton CSI. Leaflets of thin plates grew in drops with 0.05 *M* sodium cacodylate pH 6.5 and 10% PEG 8000 and 0.1 *M* magnesium or calcium acetate. Individual plates from the leaflets diffracted very poorly. Varying the pH did not improve the crystals, but decreasing the PEG content to 3–5% and the addition of glycerol yielded some crystals which were thicker and diffracted to 3.2 Å but were somewhat mosaic.

In order to conveniently produce this fragment for further crystallization trials and optimization, a construct was engineered for recombinant expression. Owing to the plasmid sequence coding at the thrombin cleavage site in the vector constructed, the sequence GSHM replaced the sequence DDAES at the N-terminus. The new construct, which we denote as RIIβ A/B, was expressed, purified and prepared for crystallization trials. In this case, the pH 6.5/PEG 8K/MgOAc condition optimal for the Δ17Δ7 fragment yielded no crystals even after varying PEG and salt. We therefore screened again with the Hampton CSI. Crystals of about 100–200 µm in their larger dimensions were now grown from sodium acetate pH 4.6 with 4–8% PEG 4000 (Fig. 4). The crystals grew in the orthorhombic space group *C*222, with unit-cell parameters a = 91.57, b = 105.91, c = 85.80 Å and diffracted to 2.3 Å at 100 K.

Our initial attempts to produce a PKA type-RIIβ regulatory subunit construct for X-ray structural studies were based on the reported deletion mutant used for RIα (Su *et al.*, 1995). We constructed an analogous deletion based on primary sequence alignment. Relatively extensive crystallization screening gave no useful leads and we were faced with expressing additional constructs with modifications to the polypeptide sequence at random or to generate biochemical information which might focus our efforts.

Limited proteolysis leads to the cleavage of a small number of peptide bonds located in the flexible surface segments of a polypeptide chain (Fontana *et al.*, 1986) and is reviewed by Neurath (1986) and Hubbard (1998). These proteolytically sensitive surface regions include junctions between relatively proteolytically resistant folded domains (Craik *et al.*, 1982). As such, this method offers the opportunity to identify folded structural domains which might be more amenable to crystallization. The crystallizability of structurally truncated subunits of proteins (Su *et al.*, 1995; Crane *et al.*, 1997; Liang *et al.*, 1997; Thompson *et al.*, 1998; Xie *et al.*, 1998; Leppanen *et al.*, 1999) points out the importance of beginning the crystallization experiment with a polypeptide of limited flexibility and proteolytic stability. We used full-length RIIβ as a starting point for limited proteolysis experiments with a panel of proteases in order to map proteolytically sensitive regions of the folded intact protein. Previous studies on the RI and RII α-regulatory subunits (Potter & Taylor, 1979; Weldon & Taylor, 1985) had identified a proteolytically sensitive 'hinge' region encompassing roughly 10–12 residues centering around the conserved RRXA of RIα. Sequence alignment of these isoforms with RIIβ identified residues 107–117 as the analogous region of RIIβ. Based on the size and N-terminal sequence of limit fragments produced in our proteolysis experiments, cleavage occurred almost exclusively in this region but extended from about residues 95–128 in RIIβ, a much more extensive 'hinge' than for RIα and RIIα. Alignment of the three sequences reveals an insert of 4–8 residues in this region for RIIβ relative to RIα and RIIα, as well as several sequence differences which might affect the ability of the various proteases to cleave. One possible explanation for our failure to obtain crystals with the Δ108 construct may be the presence of the 108–128 region which, based on our proteolysis results and the electron density for the RIα structure, would be likely to be flexible and could inhibit crystallization.

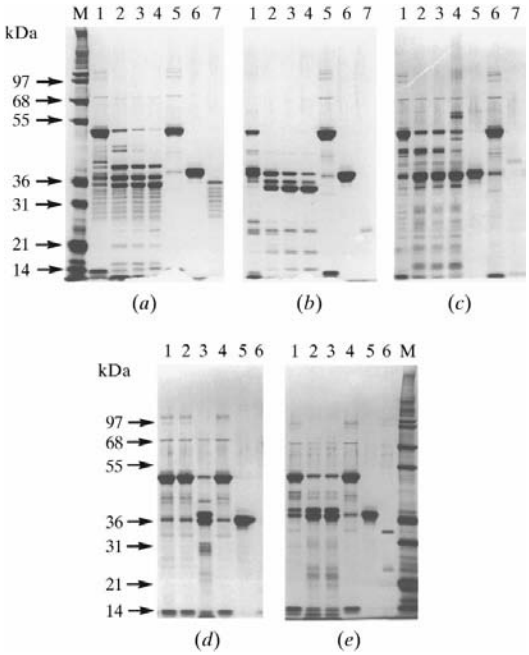

Figure 2
Limited proteolysis of full-length RIIβ. (*a*)–(*c*), lanes 1–4 are 0, 15, 30 and 60 min incubation, respectively, with (*a*) Glu-C, (*b*) Asp-N and (*c*) Arg-C. Lanes 5–7 are untreated RIIβ, RIIβ Δ108 and the respective protease. In (*d*) and (*e*), lanes 1–3 correspond to 0, 20 and 60 min incubation, respectively, with (*d*) chymotrypsin and (*e*) elastase. Lanes 4–6 are untreated RIIβ, RIIβ Δ108 and the respective protease.

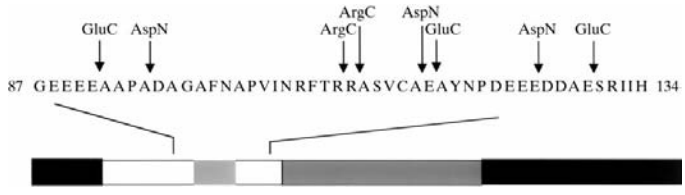

Figure 3
Proteolytic cleavages observed in the hinge region of RIIβ. Sites of cleavage by the specific proteases Asp-N, Glu-C and Arg-C were determined by N-terminal sequencing after digestion with protease and electroblotting to PVDF after SDS–PAGE. The extent of the hinge region and the cleavage sites for various proteases are shown.

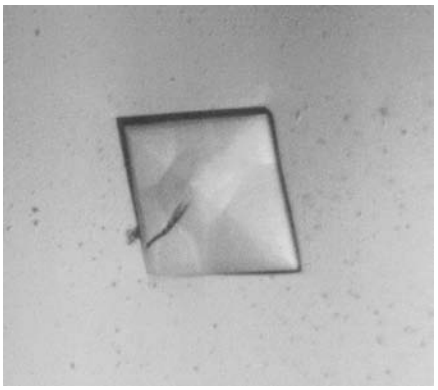

Figure 4
Orthorhombic crystal of recombinant truncated RIIβ regulatory domain. The crystal shown had dimensions of 200 × 200 × 75 µm, diffracted to 2.3 Å and had unit-cell parameters a = 91.57, b = 105.91, c = 85.80 Å.

Shabb and coworkers have reported the occurrence of a 14.4 kDa fragment generated by proteolysis during bacterial expression and purification of the recombinant RIα subunit (Shabb *et al.*, 1995). This fragment retains cAMP binding and was identified as the COOH-terminal cAMP-binding domain of RIα, beginning at Ser252. A very similar (and probably related) 16 kDa fragment was reported earlier (Rannels & Corbin, 1979) for RIIα. While some of our initial digests did produce minor fragments in the 14 kDa range, we did not characterize them further. Our proteolysis with Asp-N did not produce a fragment consistent with this cleavage event since there was no aspartic acid residue in this region of RIIβ to specify cleavage by this protease. One potential advantage of site-specific proteases is that they can avoid cleavage at sites which might be attacked by more promiscuous proteases. In this way, careful inspection of the sequence and choice of protease may increase the possibility of obtaining appropriate fragments.

pH can be an important factor in crystal growth. Clearly one mechanism for this is through effects on protein solubility. It is also thought that it may be a consequence of the effects of charge interactions in initiating contact and promoting orientation of approaching molecules (McPherson, 1999). While not a systematic study, the availability of two otherwise identical molecules with N-terminal sequence changes allowed us to observe the effects of these changes on crystallizability. Owing to the amino-acid substitutions in the A/B construct relative to the D17D7 fragment, there was a change in the charge-state possibilities at the N-terminus of the two proteins. There was also a difference of two pH units (4.6 *versus* 6.5) in the pH for crystal growth between the two versions of RIIβ fragment. The N-terminus of the D17D7 fragment with the sequence DDAES should have multiple negative charges at its optimal crystallization pH of 6.5. The RIIβ A/B fragment with the sequence GSHM substituted would have only a partially positively charged histidine at this pH and would thus present different possibilities for electrostatic interaction, possibly explaining its inability to crystallize at this pH. While this comparison does not allow us to make predictions about crystallizability, it does reinforce the well known importance of pH in crystallization of proteins and may suggest an opportunity for engineering protein termini to improve crystallization in certain cases.

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