Isolation, cloning and characterization of a low-molecular-mass purine nucleoside- and nucleotide-binding protein

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A purine nucleoside- and nucleotide-binding protein has been isolated from extracts of rat and rabbit heart, calf aortic smooth muscle and rat liver using an affinity column containing adenosine bound through the *N*⁶-position. The protein, which was eluted by adenosine, was cloned and expressed in *Escherichia coli*. The deduced amino acid sequence has a calculated *M*_r of 13693 (p13.7). The expressed protein has properties identical with the protein isolated from heart and liver, including an anomalous, apparent *M*_r of 15300, observed on gel electrophoresis. Gel filtration shows it to be a dimer. p13.7 differs by only three amino acids out of 125 from protein kinase C inhibitor 1 [Pearson, DeWald, Mathews, Mozier, Zürcher-Neely, Heinrikson, Morris, McCubbin, McDonald, Fraser et al. (1990) *J. Biol. Chem.* **265**, 4583–4591]. However, we have not been able to demonstrate inhibition of protein kinase C by physiological concentrations of

p13.7, regardless of whether it was isolated from tissue extracts or expressed in *E. coli*. p13.7 is a member of the histidine triad motif family of proteins [Séraphin (1992) *J. DNA Sequencing Mapping* **3**, 177–179]. The affinity of p13.7 for a number of different purine nucleosides and nucleotides, as measured by fluorescence titration and gel filtration, falls within the range 5–50 μ M. On the basis of these properties and its crystal structure [Brenner, Garrison, Gilmour, Peisach, Ringe, Petsko and Lowenstein (1997) *Nature Struct. Biol.* **4**, 231–238], we have coined the acronym HINT (histidine triad nucleotide-binding motif) to describe the family of proteins of which p13.7 is a member. Other proteins that bind to the affinity column have been identified as malate and lactate dehydrogenases, cAMP-binding proteins, adenosine kinase and *S*-adenosylhomocysteine hydrolase.

INTRODUCTION

We report the isolation of a purine nucleoside- and nucleotide-binding protein from soluble fractions prepared from rat and rabbit heart, calf aortic smooth muscle and rat liver by using an affinity column containing *N*⁶-(3-aminopropyl)adenosine linked to agarose via a spacer arm. All three tissues are relatively rich in the protein, which is eluted from the column in pure form by adenosine. Cloning of the protein from rabbit heart shows it to have a calculated *M*_r of 13690 (hereafter p13.7) and to be virtually identical with protein kinase C inhibitor 1 (PKCI-1) [1]. p13.7 contains the histidine triad–hydrophobic amino acid motif, H ϕ H ϕ H ϕ , where H is histidine and ϕ is a hydrophobic amino acid, that occurs in 14 different proteins and which have been called the HIT (histidine triad motif) family [2]. The histidine triad of PKCI-1 can bind zinc [3], and this was consequently assumed to be one of its functions. The product of maize gene Mz2-12 also binds zinc and it has a sequence similar to p13.7 and PKCI-1. Mz2-12 protein by itself showed little or no inhibitory activity on protein kinase C (PKC); however, in the presence of the 14-3-3 protein (a highly conserved family of 30 kDa proteins involved in PKC regulation, that has also been reported to inhibit PKC), PKCI-1 acted synergistically [4].

Using the yeast two hybrid system, with the cDNA of the ataxia-telangiectasia suppressor protein (ataxia-telangiectasia complementation protein D) from human LM217 fibroblasts as bait, Brzoska et al. [5] demonstrated an interaction of ataxia-telangiectasia complementation protein D with a protein differing by only six amino acids from the sequences of the bovine brain PKCI-1 and rabbit heart p13.7. Using a similar system, but with

the cDNA sequence for amino acids 1–317 of human PKC isoform β as probe, Lima et al. [6] found that this regulatory domain interacts with a protein also nearly identical with bovine brain PKCI-1 and rabbit heart p13.7. However, these authors too were unable to demonstrate inhibition of PKC by PKCI-1. Lima et al. [6] and Brenner et al. [7] crystallized human PKCI-1 and rabbit-heart p13.7 respectively and determined their structures, which are virtually identical. In the p13.7 structure, histidines 112 and 114 of the HIT are responsible for binding the phosphate of nucleoside monophosphates [7]. The X-ray structures showed no evidence of containing zinc [6,7], and nuclear activation measurements show that p13.7 contains < 0.1 atom zinc/100 molecules of protein. Based on the similarities of the amino acid sequences and crystal structures, we believe that p13.7 and PKCI-1 are the same proteins, minor differences being due to species differences. p13.7 shows a relative lack in specificity towards purine ribonucleosides and purine ribonucleotides, a finding which suggests that it may be a sensor for these compounds in the cell, and that, as such, it transmits binding information to downstream targets that may include the proteins with which it has been shown to interact. p13.7 binds 2'-deoxyribonucleosides and 2'-deoxyribonucleotides poorly or not at all.

EXPERIMENTAL

Materials

Synthesis of *N*⁶-(3-aminopropyl)adenosine

A mixture of 115 mg (0.4 mmol) of 6-chloropurine riboside,

Abbreviations used: HIT, histidine triad motif; HINT, histidine triad nucleotide-binding motif; PCR, polymerase chain reaction; PKC, protein kinase C; PKCI-1, protein kinase C inhibitor 1.

The nucleotide sequence of the HINT protein p13.7 has been deposited with the EMBL/DBJ/GenBank sequence database under accession number Y11175.

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0.89 g (12 mmol) of 1,3-diaminopropane and 1 ml of water was heated in a boiling-water bath for 2 h and then cooled to room temperature [8]. Ethanol (3 ml) and 5 ml of ether were added to the reaction mixture. The mixture was allowed to stand overnight at 5 °C. Crystals formed that were filtered and washed with ethanol and cold ether. The resulting material was dried in a desiccator over $\text{Mg}(\text{ClO}_4)_2$ for 3 days. The yield was 109 mg. TLC indicated a single product. The R_F values of 6-chloropurine riboside and the presumed product N^6 -(3-aminopropyl)adenosine were 0.50 and 0.0 respectively on thin-layer silica-gel plates run in methanol/ethyl acetate (1:9, v/v), and 0.75 and 0.81 respectively on thin-layer cellulose plates run in 2-methylpropanoic acid/conc. ammonia/water (66:1:33, by vol.). The product spot, but not the 6-chloropurine riboside spot, gave a yellow stain with picric acid and a brown stain with I_2 vapour.

Synthesis of adenosine affinity gel

N^6 -(3-Aminopropyl)adenosine (85 mg, 0.262 mmol) was dissolved in 50 ml of 0.16 M Hepes buffer, pH 8.4 (solution X). Affi-Gel 10 (50 ml packed volume), which contains an *N*-hydroxy-succinimide ester at the end of a spacer arm, which is linked to agarose-gel beads, was washed with 150 ml of cold propan-2-ol and 150 ml of cold water. The gel was added to solution X. The mixture was shaken gently at room temperature for 2 h [9]. The gel was filtered and suspended in 150 ml of 0.1 M Tris/HCl buffer, pH 8.5, at room temperature for 1 h to block the remaining activated ester groups. It was then washed well with water and with 50 mM Tris/HCl buffer, pH 7.5. Absorbance at 260 nm of the supernatant of the reaction mixture before and after the coupling reaction showed that 209 μmol of adenosine residue had been coupled to the gel (4.2 $\mu\text{mol}/\text{ml}$ of gel), corresponding to a coupling efficiency of 80% with respect to N^6 -(3-aminopropyl)adenosine. Other preparations in which the ratio of aminopropyladenosine to Affi-Gel 10 was changed appropriately, yielded 1.3–6.9 μmol of N^6 -(3-aminopropyl)adenosine residues/ml of gel. As will be shown below, the density of covalently linked N^6 -(3-aminopropyl)adenosine residues is crucial to the success and reproducibility of the affinity-chromatographic separations.

A 'control gel' was prepared by treating Affi-Gel 10 with Tris base only. The gel (10 ml) was washed as before and was then added to 20 ml of 0.5 M Tris/HCl buffer, pH 8.5. The mixture was allowed to stand at room temperature for 2 h with gentle shaking. The gel was filtered and washed with 50 mM Tris/HCl buffer, pH 7.5. The affinity and control gels were stored at 4 °C in a mixture of 50% (v/v) glycerol, 25 mM Tris/HCl, pH 7.5, and 0.04% (w/v) LiN_3 .

Other materials

Affi-Gel 10, acrylamide, bisacrylamide, 2-mercaptoethanol, ammonium persulphate, Bromophenol Blue, SDS and protein M_r standards were from Bio-Rad. ATP, Tris, *p*-toluenesulphonyl-fluoride, adenosine, 2',3'-dideoxyadenosine, 6-chloropurine riboside, Hepes, BSA and 1,3-diaminopropane were from Sigma. [^3H]Adenosine was purchased from NEN Research Products and purified by paper chromatography before use. TLC plates were purchased from Eastman Kodak Co. Calf aortas were obtained from a local slaughter house. Sprague-Dawley rats were from Charles River Breeding Laboratories; New Zealand albino rabbits were from Pine Acre Co. Unless otherwise indicated, molecular biologicals were from Stratagene.

Preparation of soluble fractions

All operations were performed at 4 °C. After trimming away fat and connective tissue, rat or rabbit hearts were homogenized and a high-speed supernatant of the homogenate was prepared as described previously [10], except that the homogenizing buffer was 20 mM Tris/HCl, pH 7.5. The supernatant, hereafter referred to as 'soluble fraction,' was stored at -20 °C. In the case of smooth muscle, calf aortas were slit open, pinned to a dissecting platform with the intima face up, and transverse strips of endothelial layer were peeled off with forceps before dissecting strips of the underlying media with fine forceps. The muscle was then chopped into 1 mm squares using a tissue chopper [11] and the soluble fraction was prepared as described above. Liver cytosol was prepared as described previously [12].

Cloning and expression of p13.7

Tryptic peptides were prepared from the pure p13.7 obtained by affinity chromatography of rabbit-heart soluble fraction. The peptides were separated by reversed-phase HPLC [13] and sequenced by the Brandeis sequencing facility. Three peptides so obtained had sequences identical with PKCI-1 [1]. The sequence of PKCI-1 was obtained by classical peptide sequencing and not by cloning and sequencing the corresponding cDNA. Accordingly, we prepared sense and antisense primers based on back-translations of two of our sequenced peptides. The primers were used for amplification by the polymerase chain reaction (PCR) of the sequence between the two peptides using a rabbit-heart cDNA library contained in Lambda ZapII vector (Stratagene). This yielded a 276 bp band of DNA. The PCR product was labelled with random ^{32}P -labelled primers in another round of PCR. The rabbit-heart cDNA library was screened with the resulting probe. Positive clones were converted from phagemid into plasmid in XL1-Blue cells (Stratagene) as described by Bullock et al. [14] and sequenced using Sequenase Version 2.0 DNA Sequencing Kit (DuPont-NEN Research Products, Boston, MA, U.S.A.).

The cloned sequence was subcloned into expression vector pSGA02 [15] as follows. An *Nde*I restriction site (5'-CATATG-3') was created at the initiating ATG codon and an *Xho*I site (5'-CTCGAG-3') was created after the terminating codon of the p13.7 cDNA by site-directed mutagenesis using the method of Kunkel et al. [16]. p13.7 was expressed at 37 °C and the cells were harvested, extracted and centrifuged as described previously [15]. The resulting extract could be stored frozen at -20 °C at this stage.

Measurement of purine nucleoside and nucleotide binding by fluorescence titration

p13.7 contains a single tryptophan. The fluorescence of p13.7 changes upon binding purine nucleosides and nucleotides. Fluorescence titrations were conducted using the method described by Elalaoi et al. [17]. The excitation and emission wavelengths used were 295 and 375 nm respectively. The inner filter effect was corrected for by conducting control titrations using *N*-acetyl-tryptophanamide.

RESULTS

Isolation of p13.7 from various organs by adenosine affinity-column chromatography

Soluble fraction prepared from rat heart was applied to an N^6 -(3-aminopropyl)adenosine affinity column (bed volume 10 ml) containing 1.3 μmol of adenosine residues/ml of gel. After the

Figure 1 Affinity chromatography of rat-heart soluble fraction

(Upper panel) The column contained 10 ml of adenosine affinity gel [$1.3 \mu\text{mol}$ of N^6 -(3-aminopropyl)adenosine/ml of gel]. A total of 150 ml of soluble fraction (540 mg protein), prepared as described in the Experimental section, was applied to the column, and fractions of 7.5 ml were collected. The column was then washed with 65 ml of 20 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl (buffer A), and fractions of 6 ml were collected. The column was then washed with 90 ml of buffer A containing 200 μM cAMP, and fractions of 6 ml were collected. Finally the column was eluted with 100 ml of buffer A containing 200 μM adenosine, and fractions of 4.3 ml were collected. Fractions were pooled as indicated by the solid, horizontal bars. The pooled fractions were concentrated and dialysed by vacuum dialysis and subjected to SDS/PAGE on a 12% (w/v) gel, with the result shown in the lower panel. For ○, use left ordinate; for ●, use right ordinate. (Lower panel) Soluble proteins from rat heart that bind to adenosine affinity gel. The protein pattern of the soluble fraction (SF) used as starting material is very similar to that of the soluble fraction minus binding proteins (SF-BP) obtained after passing SF through the affinity column. Lanes marked A_I , A_{II} and A_{III} show the protein patterns in pooled fractions obtained from washing the column with buffer A. Lanes marked cAMP and ado show the protein patterns in pooled fractions obtained from washing the column with cAMP and adenosine respectively. M_r markers ($\times 10^{-3}$) were: phosphorylase (97.4), BSA (66.2), ovalbumin (45), carbonic anhydrase (29), soya bean trypsin inhibitor (21.5), myoglobin (17) and lysozyme (14.3).

soluble fraction had passed through the column, the column was washed with 20 mM Tris/HCl buffer (pH 7.5)/0.15 M NaCl (buffer A) until the protein concentration of the effluent had dropped to approx. 2 $\mu\text{g}/\text{ml}$. The column was then eluted with 80 ml of buffer A containing 200 μM cAMP. Finally, the column was eluted with buffer A containing 200 μM adenosine. The protein elution profile of one of these experiments is shown in Figure 1(A). The soluble fraction that passed through the column was pooled and is hereafter referred to as 'soluble

Figure 2 Soluble proteins from rat liver that bind to adenosine affinity column

An experiment similar to that described in Figure 1 was run, except that the soluble fraction was from liver. The protein patterns of the soluble fraction (SF) applied to the column and the soluble fraction minus binding proteins (SF-BP) obtained after passing SF through the column were visually indistinguishable. Note that the intensity of staining of the two proteins eluted by cAMP plus buffer is reversed when compared with heart.

fraction minus binding proteins'. The eluate obtained from washing the column with buffer A was pooled in three lots (A_I , A_{II} and A_{III}). Eluates obtained from washing the column with cAMP and adenosine were pooled as shown in Figure 1(A).

With the exception of A_I , the pooled fractions were each concentrated and dialysed before being subjected to SDS/PAGE. Comparison of the gel electrophoresis pattern for the soluble fraction with that for the soluble fraction minus binding proteins shows no obvious differences (Figure 1B). The gel pattern for A_I shows that it represents the residual soluble fraction minus binding proteins. The presence of certain bands and not of others in A_I indicates that some fractionation took place during this early stage of washing the column with buffer A. Pooled A_{II} fractions yielded only two protein bands with subunit M_r values of 37000 and 35000. Pooled A_{III} fractions yielded the same bands, except that the 35000 Da band was much more enriched. Pooled fractions of proteins eluted by cAMP yielded two weak bands with subunit M_r values of 46000 and 43000, with the former more intense than the latter. Lastly, pooled fractions of protein eluted by adenosine yielded a single, intense band. Comparison with M_r standards indicated that the native and expressed proteins have an apparent M_r of 15300. However, sequencing of the corresponding cDNA (see below) yielded a calculated M_r of 13693. Anomalous behaviour on gel electrophoresis was also observed for PKCI-1 by McDonald et al. [18], who reported it to have a M_r of 17000.

In other experiments with soluble fraction from heart, the elution with cAMP was omitted and the column was washed with buffer A, followed by buffer A plus 200 μM adenosine. Three proteins were now eluted by adenosine; a major component with a M_r of 46000, a minor component with an M_r of 43000, and another major component with an M_r of 15300 (results not shown). Other experiments showed that the 46000 and 43000 Da proteins were also eluted by buffer A containing either 200 μM 5'-deoxy-5'-chloroadenosine or 200 μM N^6 -cyclohexyladenosine. Soluble fraction prepared from rat heart was also applied to a column packed with 'control gel' (see the Materials section) and

Table 1 Proteins isolated by chromatography on an *N*⁶-(3-aminopropyl)adenosine affinity column

Yield is quoted as the percentage of total cytosolic protein. Results are means of three determinations. In the case of closely adjacent pairs of bands, the recovery is quoted for both together. n.d. = not done.

Source of cytosol	Eluting agent ...		200 µM cAMP plus buffer A		200 µM Adenosine plus buffer A	
	Buffer A	Yield (%)	10 ⁻³ × <i>M_r</i>	Yield (%)	10 ⁻³ × <i>M_r</i>	Yield (%)
Rat heart	35*	0.14	43*	0.01	13.7†	0.07
	37		46			
Rat liver	35	0.35	43	0.13	13.7	0.07
			46*			
Calf aorta smooth muscle	37	0.27	43	0.05	13.7	n.d.
			46*			

* Major band.
† *M_r* was 15300 by SDS gel electrophoresis.

elution was carried out as described above. Under these conditions no proteins were found in pooled eluate fractions labelled A_{II}, A_{III}, cAMP and adenosine in Figure 1B (results not shown). An experiment similar to that described in Figure 1 was performed with a soluble fraction prepared from rat liver. The SDS/PAGE pattern of the pooled fraction obtained from the affinity column is shown in Figure 2. Buffer A eluted from the column a single band with an *M_r* of 35000 Da; whereas in the case of heart it eluted two bands with *M_r* values of 35000 and 35700 Da. The proteins eluted by cAMP had *M_r* values similar to those found in heart; however, the intensity of the two bands was reversed when compared with the bands from heart. The protein eluted by adenosine ran at the same position as p13.7 from heart.

A modification of the above protocol showed that after the affinity column has been washed with buffer A, the 43000 and 46000 Da proteins are readily eluted with 30 µM adenosine; p13.7 is then readily eluted in pure form with 200 µM adenosine. This modification was used to isolate pure p13.7 from rabbit heart. p13.7 isolated from rat heart, liver and smooth muscle, as well as from rabbit heart, behaved identically on gel electrophoresis.

The yields of the various proteins obtained by affinity chromatography of extracts of heart, liver and smooth muscle are shown in Table 1. Assuming that the concentration of cytosolic proteins in rat liver is 200 mg/ml, it can be calculated that the concentration of p13.7 is approx. 11 µM.

Identification of other proteins retained by adenosine affinity column

The proteins retained weakly by the affinity column and eluted with cAMP or low concentrations of adenosine have been identified as follows. Fractions A_{II} and A_{III} from liver, which showed a single band at *M_r* 35000 (Figure 2), contained malate and lactate dehydrogenases of high specific activity. Fraction A_{III} from heart showing bands at *M_r* 35000 and 37000 (Figure 1B) also contained malate and lactate dehydrogenases, but of lower specific activity. Fractions eluted with 200 µM cAMP or 30 µM adenosine, which showed a band at *M_r* 43000, contained adenosine kinase of high specific activity, whereas the band at *M_r* 46000 was most probably *S*-adenosylhomocysteine hydrolase. The methods used to identify these enzymes will be supplied on request.

Figure 3 Deduced amino acid sequence

Peptide sequences obtained from isolated rabbit protein, redundant primers made for the PCR reaction, cDNA sequence obtained from clone obtained using the PCR product as probe, and deduced amino acid sequence are shown. The peptides sequenced are indicated by broken lines. The redundant primers made for the PCR reaction were based on peptides 16 and 24 and are shown in lower case letters, where n = a, t, g and c; m = c and a; y = c and t; h = c, t and a; r = g and a; s = c and g; w = t and a. The deduced amino acid sequence has three differences from the amino acid sequence of PKC1-1, namely F33Y, H59Y and A69D, which are shown in bold letters. The nucleotides substituted by site-directed mutagenesis to create an *Nde*I restriction site (positions -3 to +3) and an *Xho*I restriction site (positions 383-388) are underlined. These mutations were made to insert the cDNA into vector pSGA02.

Effect of concentration of immobilized adenosine

The experiment reported in Figures 1 and 2 has been repeated 15 times with similar results. The affinity gel used in these experiments contained 1.3 µmol of adenosine residues/ml of gel. When we used a different batch of affinity gel, which contained 4.2 µmol of adenosine residues/ml of gel, we observed that 200 µM cAMP did not elute the *M_r* 46000 and 43000 proteins completely as before; however, these proteins and p13.7 were eluted by 200 µM adenosine. Thus it appears important to have the correct density of adenosine residues on the affinity gel in order to obtain

Figure 4 Purification of p13.7 expressed in *E. coli*

Protein was purified as described in the Results section. The yield was typically 100 mg of pure protein/litre of cell culture. Lane 1, M_r standards; lane 2, soluble fraction; lane 3, fraction obtained after passing soluble fraction through the affinity column; lanes 4–8, successive fractions from elution of column with 200 μ M adenosine; lane 9, M_r markers ($\times 10^{-3}$): BSA (66.2), ovalbumin (45), carbonic anhydrase (29), myoglobin (17) and lysozyme (14.3).

reproducible separations. As the density of covalently linked adenosine is increased, oligomeric adenosine-binding proteins may bind to two adenosine residues linked to the gel, which would make the bound proteins harder to elute.

Cloning and sequencing of p13.7

Results of the cloning and sequencing of p13.7 are shown in Figure 3. p13.7 differs from the PKCI-1 of Pearson et al. [1] in the substitutions F33Y, H59Y and A69D.

Isolation of p13.7 after expression in *Escherichia coli* using adenosine affinity chromatography

The supernatant obtained from the *E. coli* extract containing the expressed p13.7 was applied to the adenosine affinity column (bed volume 25 ml) as described above. The column was washed with buffer A until the protein concentration being eluted was less than 2 μ g/ml. The column was then eluted with buffer A containing 200 μ M adenosine. Fractions of 4.9 ml were collected. This yielded a single protein peak which contained pure p13.7 as judged by SDS/PAGE (Figure 4). The pooled fractions containing p13.7 were dialysed and concentrated. The yield of pure p13.7 from 1 litre of culture containing approx. 4 g wet weight of *E. coli* was approx. 100 mg (mean of three preparations).

Specificity of nucleoside and nucleotide binding measured by elution from adenosine affinity column

In addition to being eluted by adenosine, p13.7 was eluted from the adenosine affinity column by the following ligands (listed in the order in which they eluted p13.7): 1, N^6 -ethenoadenosine, 5'-deoxyadenosine, 8-bromoadenosine, GMP, guanosine and IMP. These ligands were used in the form of linear gradients (0–400 μ M) in buffer A (Figure 5). p13.7 was not eluted from the adenosine affinity column by buffer A containing 200 μ M of one of the following: AMP, ATP, NAD, 2'-deoxyadenosine, 2',3'-dideoxyadenosine, inosine, cytidine, thymidine and adenine. p13.7 was also not eluted by prolonging the elution with 200 μ M cAMP in buffer A, or by a saturated solution of *p*-nitrobenzylthioinosine in buffer A, or by buffer A alone. These results do not rule out that one or more of these substances are able to elute p13.7 at concentrations > 200 μ M. Although elution of p13.7 from the adenosine affinity column permits assessment of relative affinities of these compounds, it is difficult to use this technique

Figure 5 Elution of p13.7 from adenosine affinity column by various ligands

p13.7 (1 mg) was dialysed against 20 mM Tris/HCl buffer, pH 7.5, and 0.15 M NaCl (buffer A) to remove adenosine. It was then applied to the adenosine affinity column (1.5 cm diameter, 10 ml) at 0.5 ml/min and the column was washed with 50 ml of buffer A. The column was eluted with a linear gradient (0–400 μ M) of ligand in 150 ml of buffer A. ●, 5'-Deoxyadenosine; ○, ethenoadenosine; ■, adenosine; □, GMP; ◆, IMP.

for measuring absolute affinities, because the effective concentration of the immobilized adenosine is not known. Actual affinities were measured by fluorescence titration.

Affinities for nucleosides and nucleotides measured by fluorescence titration

Fluorescence titrations showed that purine nucleoside monophosphates quenched the fluorescence of p13.7, whereas purine nucleosides enhanced its fluorescence. 2'-Deoxyadenosine, 3'-AMP, ADP and ATP had no effect. Results of a fluorescence titration with one of the ligands tested, 8-bromoadenosine, are shown in Figure 6 in the form of a Scatchard plot. All of the

Figure 6 Scatchard plot of fluorescence titration of p13.7 with 8-bromo-AMP

The concentration of p13.7 was 25 μ g/ml (1.82 μ M with respect to monomer), the buffer was 50 mM Hepes, pH 7.4, and the temperature was 25 °C. The linear regression line yields a stoichiometry of 0.95:1 ($r^2 = 0.99$) and a K_d of 5 μ M. v stands for mol of ligand bound/p13.7 monomer. L is the concentration of free ligand.

Table 2 Affinity of p13.7 for some nucleosides and nucleotides

The dissociation constants were calculated from fluorescence titrations as described by Elalaoui et al. [17]. The effect of ligand on fluorescence is indicated by E, enhancement; Q, quenching; and N, none. The data are from single-titration curves, one of which is shown in Figure 6.

Ligand	K_d (μM)	Effect on fluorescence
8-Bromo-5'-AMP	5	Q
5'-Deoxyadenosine	8	E
5'-AMP	12	Q
5'-GMP	24, 68*	Q
5'-IMP	42	Q
Adenosine	53	E
Inosine	50–100†	E
2'-Deoxyadenosine	—	N
3'-AMP, ADP, ATP	—	N

* Required large corrections; showed anomalous behaviour, suggestive of two binding sites.
† Weak enhancement.

ligands tested showed a 1:1 binding stoichiometry based on the molecular mass for the monomer of 13700.

The dissociation constants for various ligands calculated from the titration data are given in Table 2. There appears to be no simple relationship between the apparent K_d values and the order of elution by purine nucleosides and nucleotides from the affinity column (Figure 5). Binding of adenosine to p13.7 from rat heart was also measured by the method of Hummel and Dreyer [19]. The results yielded a K_d of 32 ± 11 (S.D.) μM .

Molecular mass

p13.7 prepared from rat heart extracts or expressed in *E. coli* from rabbit heart cDNA was subjected to gel filtration in the presence and absence of molecular-mass markers. Both types of preparation ran slightly more slowly than carbonic anhydrase and had an apparent M_r of 30000 (results not shown). This is consistent with the protein in solution being a dimer. The crystal structure also shows it to be a dimer [7]. p13.7 contained < 0.1 atom of Zn/100 molecules of monomer.

DISCUSSION

The sequence of p13.7 shows that it is virtually identical with PKCI-1 [1,3] and both p13.7 and PKCI-1 are dimers. We believe that the two are the same protein and that the small differences reflect species differences only. The yield of p13.7 from rat heart and liver was approx. 4.5 nmol/g wet weight (calculated from the data in Table 1). The protein occurs largely or exclusively in the cytosol. Assuming that cytosolic water accounts for approx. 40% of the wet weight and that our recovery was quantitative, this is equivalent to a p13.7 concentration of 11 μM .

The significance of the broad purine nucleoside and nucleotide specificity of p13.7 is not obvious. One possibility is that p13.7 acts as a sensor of the concentrations of these substances. Another is that the nucleosides and nucleotides mimic some other, possibly more potent, ligand. The function of p13.7 may be analogous to that of the R subunit of cAMP-dependent protein kinase A, but with a different target, which may itself influence the specificity towards ligand. It is noteworthy that nucleosides enhance whereas nucleotides quench the fluorescence

of p13.7 (Table 2). The crystal structure of p13.7 shows only minor conformational differences between p13.7 in the presence and absence of bound nucleoside or nucleotide ligand [7]. However, ligand binding itself may result in a change of the p13.7 surface sufficient to cause it to interact, or to prevent it from interacting, with a downstream target. A possible model is the interaction of the fungal metabolite FK506 with peptidyl-prolylisomerase [also known as FK506-binding protein (FKBP12)], which inhibits the isomerase activity. However, the chief target of the FK506–FKBP12 complex is calcineurin, a serine/threonine phosphatase that is powerfully inhibited by the complex. Neither FK506 nor FKBP12 alone is inhibitory. The inhibition occurs even though conformational changes in FKBP12 are minor when it binds FK506; however, the new surface makes a much better fit with its target [20].

We failed to observe PKC inhibition by p13.7. These negative results, which have also been reported by others, indicate that the function of p13.7 is not simply that of a PKC inhibitor, as originally stated by McDonald and Walsh [21]. Such negative results do not rule out an involvement of p13.7 in regulating PKC in a manner that involves additional factors, which may have been present in the preparations of PKCI-1 of McDonald and Walsh [21], obtained using reversed-phase chromatography of brain extracts in the presence of high concentrations of EDTA.

Adenosine is produced in response to metabolic events that lower ATP and raise the AMP and ADP concentrations and thereby activate 5'-nucleotidase I. This isoform of 5'-nucleotidase prefers AMP as the substrate over IMP and is activated by ADP [22]. Under normoxic conditions, rat heart contains 2.4 nmol of adenosine/g fresh weight. The adenosine content rises steeply once the oxygen level drops below approx. 10% of saturation [23], reaching approx. 18 nmol/g fresh weight after 25 s of anoxia [24]. Adenosine also rises in response to chemical work, such as the conversion of acetate into acetyl-CoA by acetyl-CoA synthetase, which occurs during ethanol metabolism and haemodialysis with fluid containing acetate as the major anion [25]. Adenosine can leave and enter cells via a nucleoside transporter. The specific activity of this transporter in heart is not known, but efflux of adenosine is rate-limiting [26], and it is a complex matter to determine the distribution of adenosine between the intra- and extracellular spaces [27]. Ignoring that efflux of adenosine is rate-limiting for the moment, and assuming that adenosine is distributed rapidly between these spaces and that 75% of the tissue wet weight is water, one obtains average concentrations of 3.2 μM and 24 μM adenosine in normoxic heart and heart that has been anoxic for 25 s respectively. Using the K_d of 53 μM for p13.7 (Table 2), it can be calculated that in the normoxic heart, 5% of the p13.7 contains bound adenosine. After 25 s of anoxia, 57% of the p13.7 contains bound adenosine, an 11-fold increase. If adenosine efflux is rate-limiting, the intracellular concentration of adenosine will be higher and more p13.7 will contain bound adenosine.

AMP concentrations also change in response to hypoxia and increased work load. The actual values differ greatly depending on which organ is considered. They fall in the region of 0.24 and 1.1 μM for rat hind limb between rest and exercise respectively [28], suggesting that in this instance changes in [AMP] will have little influence on p13.7. They fall in the region of 20 and 133 μM for rat brain before and after 2 h of ischaemia respectively [29], a range that spans the $K_{d,app}$ of p13.7 for AMP (Table 2). They fall in the region of 200 and 625 μM for perfused guinea-pig heart between control and no substrate in the perfusate respectively [30], and 160 and 440 μM for perfused rat liver in the fed and fasted state respectively [31], which are concentrations well

above the $K_{d,app}$ of p13.7 for AMP. (These concentrations were recalculated from the original data, which were expressed in terms of $\mu\text{mol/g}$ dry weight.)

We believe that p13.7 is a regulatory protein whose properties depend on whether or not it is occupied with nucleoside or nucleotide. The above calculations indicate that adenosine and AMP concentrations vary in a manner and over a range in which they could serve as effectors of p13.7.

The isolation of p13.7 described here is much simpler than the isolation procedure for PKCI-1. In addition, our purification procedure yields adenosine kinase with a purity and specific activity (results not shown) similar to those obtained by much lengthier methods [32].

We reported previously the isolation of a 28 kDa protein by affinity chromatography on a column containing 5'-amino-5'-deoxyadenosine linked to agarose through the 5'-amino group [33]. Comparison of partial peptide sequences of this protein show that they bear no relationship to p13.7. An adenosine affinity column was used by Bembenek [34] to isolate two membrane proteins from bovine brain. In addition to being eluted by adenosine, these proteins were also eluted by cAMP, AMP, ADP and ATP, but not by inosine, IMP and GMP. This nucleotide specificity is entirely different from that described here and shows that the proteins isolated by Bembenek differ from p13.7.

Sequence homology searches show that p13.7 is a member of the HIT super-family of proteins, which possesses a strictly conserved histidine triad. In p13.7 this is ϕ -X-H₁₁₀- ϕ -H₁₁₂- ϕ -H₁₁₄- ϕ - ϕ , where ϕ stands for hydrophobic amino acids [2,7]. Also, strictly conserved in HIT proteins are Phe-19, His-51 and Leu-53 of p13.7, and many other positions have largely conserved amino acids. (The numbering is that of Figure 3.) HIT proteins fall into two branches, one of which we have named histidine triad nucleotide-binding motif (HINT), or histidine triad nucleotide-/nucleoside-binding proteins [7], and the other of which has been named FHIT, for chromosome 3 fragile site locus histidine triad proteins [35].

The HINT family is at present known to encode 14 proteins. Mammalian HINT proteins are nearly identical in a core of 102 amino acids, but several predicted proteins from bacteria and archaeobacteria have greater identity with HINT than their lower animal and non-animal eukaryotic counterparts [7]. The FHIT family at present consists of two diadenosine polyphosphate hydrolases [35–37] and an open reading frame from *Saccharomyces cerevisiae* (GenBank accession no. U28374) that can be predicted to have this activity. FHIT proteins possess 18 conserved residues not found in the HINT family. In addition, FHIT proteins have an extension at the C-terminus that contains eight strictly conserved amino acids. Possible relations of purine nucleoside-binding sites between HINT and FHIT are discussed elsewhere [7].

This work was supported by National Institutes of Health Grant GM-07261. We thank Martha McDonald, Hans Ghayee and Vipin Suri for performing some of the

experiments, and Dr. Ilhan Olmez, Massachusetts Institute of Technology, for performing the zinc analyses.

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