Identification and characterization of a novel A-Kinase-anchoring protein (AKAP120) from rabbit gastric parietal cells

Daniel T. DRANSFIELD, Jennifer L. YEH, Alvin J. BRADFORD and James R. GOLDENRING*

Institute of Molecular Medicine and Genetics, Departments of Medicine, Cellular Biology and Anatomy and Surgery, Medical College of Georgia, and the Augusta Veterans Affairs Medical Center, Augusta, GA 30912-3175, U.S.A.

The type-II cAMP-dependent protein kinase (A-Kinase) partitions primarily into the particulate fraction in gastric parietal cells. Localization of this kinase to particular subcellular domains is mediated through the binding of the regulatory subunit (R_{II}) dimer to A-Kinase-anchoring proteins (AKAPs). Using a [32 P] R_{II} overlay assay, we have screened a rabbit gastric parietal cell cDNA library and have isolated a single R_{II} -binding protein clone. Sequence analysis revealed an open reading frame coding for 1022 amino acids (AKAP120). Recombinant fragments of the full-length clone were prepared and the R_{II} -binding region mapped to an area between amino acids 489 and 549. This area contained a putative α -helical R_{II} -binding region between amino

acids 503 and 516. Incubation of [32 P] R_{II} with a synthetic peptide of AKAP120-(489–522) completely inhibited the binding of [32 P] R_{II} to the recombinant AKAP120 fragments that demonstrated R_{II} binding. *In vitro* R_{II} -binding affinity studies indicated a high-affinity interaction between AKAP120 and R_{II} with a K_{app} between 50 and 120 nM for the three recombinant fragments that bound [32 P] R_{II} . RNase-protection analysis revealed that AKAP120 is a widely distributed protein, with the highest levels of mRNA observed in gastric fundus. The presence of this novel high-affinity AKAP in gastric parietal cells suggests that it may regulate R_{II} subcellular sequestration in this cell type.

INTRODUCTION

The primary function of the second-messenger cAMP is to activate A-Kinase. Two major types of A-Kinase exist (I and II), distinguished by dissimilar regulatory subunits, designated R_I and R_{II} respectively. In most cells, the type-I A-Kinase is primarily cytosolic, whereas the type-II A-Kinase segregates into the particulate fraction, associating with a variety of intracellular organelles and cytoskeletal elements [1]. Site-specific localization of the type-II A-Kinase occurs through the interaction of R_{II} with specific R_{II} -binding proteins, referred to as A-Kinase-anchoring proteins (AKAPs) [1–3]. Subcellular sequestration of second-messenger-responsive enzyme systems may be responsible for tissue-specific patterns of cellular activation. Cell-specific A-Kinase function may be determined by AKAPs which tether the holoenzyme to particular subcellular sites. Such localization of A-Kinase would dictate which substrates were most accessible to the catalytic subunit on activation by cAMP [1,2]. Multiple putative AKAPs, ranging in size from 34 to 300 kDa, have been observed in a variety of tissues using a [32 P] R_{II} electroblot overlay procedure [4]. Although all known AKAPs possess an α -helical R_{II} -binding region, their overall structural similarity is minimal [1].

Histamine-stimulated acid secretion from gastric parietal cells is mediated by an increase in intracellular cAMP [5]. Increases in cAMP in rabbit parietal cells are accompanied by activation of the cytosolic type-I A-Kinase, as reflected by a decrease in [32 P]azido-cAMP photoaffinity labelling of R_I [5]. In addition, histamine stimulated the dephosphorylation of the particulate-associated R_{II} in gastric parietal cells [6]. Gjertsen et al. [7] have documented that, if the A-Kinase is actively phosphorylating substrates, the bulk of autophosphorylated R_{II} may be dephosphorylated within 1 min. Therefore histamine-induced dephos-

phorylation of R_{II} probably reflects the dissociation of the inactive holoenzyme complex (i.e. activation) of the type-II A-Kinase. The type-II A-Kinase in gastric parietal cells is predominantly particulate [6], and we have recently identified several gastric R_{II} -binding proteins with molecular masses of 78, 130 and 140 kDa [8]. The purpose of this study was to identify AKAPs in gastric parietal cells by screening a rabbit parietal cell cDNA library, and to examine their molecular characteristics to understand better their possible role in the histamine-stimulated acid-secretory response.

We now describe the cloning and molecular characterization of a novel AKAP, AKAP120, which contains a high-affinity R_{II} -binding domain as well as three C-terminal leucine-zipper motifs. AKAP120 represents the first AKAP that is enriched in gastrointestinal tract tissues.

EXPERIMENTAL

Materials

[γ - 32 P]ATP, [α - 32 P]CTP and [α - 32 P]UTP were purchased from New England Nuclear (Boston, MA, U.S.A.). A-Kinase catalytic subunit, DNA fmol sequencing kits, PolyATtract System kits and restriction enzymes were purchased from Promega (Madison, WI, U.S.A.). Custom oligonucleotides were prepared by either Cruachem (Atlanta, GA, U.S.A.) or Integrated DNA (Coralville, IA, U.S.A.). Pfu polymerase and PCRscript cloning kits were obtained from Stratagene (La Jolla, CA, U.S.A.). All pET vector plasmids and associated reagents were purchased from Novagen (Madison, WI, U.S.A.). Isopropyl thiogalactoside (IPTG) and Sequenase DNA sequencing kits were obtained from USB (Cleveland, OH, U.S.A.). Rifampicin was purchased from Sigma (St. Louis, MO, U.S.A.). Protogel for SDS/PAGE was obtained from National Diagnostics (Atlanta, GA, U.S.A.). Ni^{2+} -chelate

Abbreviations used: A-Kinase, cAMP-dependent protein kinase; AKAPs, A-Kinase-anchoring proteins; IPTG, isopropyl β -D-thiogalactopyranoside; MAP-2, microtubule-associated protein-2.

* To whom correspondence should be addressed.

The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL and DDBJ Nucleotide Sequence Databases under accession number U26360.

A.

	GGCACGAGGGAAAACTAGAGTACAGTGTCAAGCTGAAAAAGTACGTGATGACCTTCAGAAACAAGTGAAAGCTCTAGAAATAGATGTTGAGGAACAAGCTCTGAGCTTTATAGAGCTGGAACAAGAAAAAATGCTGAACATA	
1	ATGGATTAAAGACACAAACCAAGCCCTGGAAAAACAGTTAGAAAAATAGAAAAATTTTAGTAGAGCAGGCCATTGATAGAGAACAT	90
1	MetAspLeuArgGlnGlnAsnGlnAlaLeuGluLysGlnLeuGluLysMetArgLysPheLeuAspGluGlnAlaIleAspArgGluHis	30
91	GAGAGAGATGTATTCCACAGGAGATACAAAGCTAGACAGCAACTTAAGCTTGTTCTCGATTCCAGCCAATCAGTGAACATCAAACT	180
31	GluArgAspValPheGlnGlnGluIleGlnLysLeuGluGlnGlnLeuLysLeuValProArgPheGlnProIleSerGluHisGlnThr	60
181	AGAGAGGTTGAACAATTAAACAATCATCTGAAGGAAAAACAGACAAGTGCAGTGAACTTTTGCTGTCTAAAGAGCAGCTACAGAGGGAC	270
61	ArgGluValGluGlnLeuThrAsnHisLeuLysGluLysThrAspLysCysSerGluLeuLeuLeuSerLysGluGlnLeuGlnArgGlu	90
271	GTACAGGAACCGAATGAAGAAATTGAGAACTAGAGTGCAGAGTAAGGAACTGGAGCAAGCTTGCTATCAGTGCAGACACTTCCAAA	360
91	ValGlnGluArgAsnGluGluIleGluLysLeuGluCysArgValArgGluLeuGluGlnAlaLeuLeuSerValGlnThrLeuSerLys	120
361	AGGTGAGGACCCGAAACAGTTTCGGAGCTGTAGAACCTAAAGCAGAGTTGTGCTTAGAAGTACAATTGACAGCTGAGCAGATGCCATA	450
121	ArgTrpArgThrArgAsnSerPheGlyAlaValGluProLysAlaGluLeuCysLeuGluValGlnLeuGlnAlaGluArgAspAlaIle	150
451	GACAGAAAGGAGAGAGATCACAACTTAGAAGAACAAATTAGAGCAGTTTAGAGAGAACTGGAAAAATAAGAAATGAGGAGCTCCAGCAG	540
151	AspArgLysGluLysGluIleThrAsnLeuGluGlnGlnPheArgGluGluLeuGluAsnLysAsnGluValGluGlnGln	180
541	CTACATATGCACTAGAGATACAGAAAAAGGATCCACTACTCGCTGCAAGAACTTGAACAAGAAAAAATTTATTAAGGATGAATG	630
181	LeuHisMetGlnLeuGluIleGlnLysLysGluSerThrThrArgLeuGlnGluLeuGlnGluAsnLysLeuPheLysAspGluMet	630
631	GAGAAACTGGGAATTTCCGATAAGCGAATCTGATGCTGTATCTCCTCAGGACCAACAGGTGCTGTGTTGAAAAATTGTGCTCAAAATAACAT	720
211	GluLysLeuGlyPheAlaIleLysGluSerAspAlaValSerProGlnAspGlnGlnValLeuPheGlyLysPheAlaGlnIleIleHis	240
721	GAAGAGAGGTAGAAATTCACGATTAATAGCAAAATTTAAACTTCAACAGCAACTTAAATTTCAACAGATTAACAGGTCATTGAA	810
241	GluLysGluValGluIleAspArgLeuAsnGluGlnIleIleLysLeuGlnGlnGlnLeuLysIleThrThrAspAsnLysValIleGlu	270
811	GAAGAAAAATGAATGTAGAGGATCTTGAAGCCAGATAGAAATGTTTATGAGTGATCAAGAACCTGTGAGGAAAAATAGAGAAAGAGAA	900
271	GluLysAsnGluLeuIleArgAspLeuGluGlnIleGluCysLeuMetSerAspGlnGluArgValArgLysLysAspGluGluGln	300
901	ATAGAGCAGCTCAATGAAGTGATGAAAACTTCAACAGGAATTTGCAAAATATTGACCAAGAACCTCCGTGGACCCAGCTTCCCTATCA	990
301	IleGluGlnLeuAsnGluValIleGluLysLeuGlnGlnGlnAlaAsnIleAspGlnLysThrSerValAspProSerSerLeuSer	330
991	GAGGAAGCAGACAGCTTTGAAGCATCAGTTGGATAAGGTGATCGCAGAAAAGCTGGCTTGGAGCATCAAGTCGAACCACTAAGAGAA	1080
331	GluGluAlaAspSerLeuLysHisGlnLeuAspLysValIleAlaGluLysLeuAlaLeuGluHisGlnValGluThrThrAsnGluGlu	360
1081	ATGGCTGTACGAAAAATGTACTTAAAGAAACCAATTTTAAATGAATCAGCTAACCAAGAATTTATGACGCTTAAAGAGAGAACCTGAA	1170
361	MetAlaValThrLysAsnValLeuLysGluThrAsnPheLysMetAsnGlnLeuThrGlnGluLeuCysSerLeuLysArgGluGlu	390
1171	AAGATGAGAGAAATCCAGAGCTTACAGAGAAAAAGTGTAAATCATGAGTGTGGGTGATCTGAGCAAGACAACTGAGATGAGCTTAATC	1260
391	LysMetGluArgIleGlnSerValProGluLysSerValAsnMetSerValGlyAspLeuSerLysAspLysProGluMetAspLeuIle	1420
1261	CCTACTGAGGATGCTTTAGCAGAGCTGAAAACTCAGACTCAGCTCAGATCTTCAGAGAAGACGCAAGTTTTCCTTGAGTAGTTTGAA	1350
421	ProThrGluAspAlaLeuAlaGlnLeuGluThrGlnGlnLeuArgSerSerGluGluSerSerLysValSerLeuSerSerLeuGlu	450
1351	ACAAAGTTGCTTTCAGCTGGAGAGCAGCTTTAGCACAAAGGACTTGGAACTTACCAAGTGTATTAAGCAAAATCAGGACATCGCAGAGCAA	1440
451	ThrLysLeuLeuGlnLeuGluSerThrValSerThrLysAspLeuGluLeuThrGlnCysTyrLysGlnIleGlnAspMetArgGluGln	480
1441	GGCCGATCTGAAACAGAGATGCTGCAAAACGAAGATTGTAAAGCTTCAGAAAGTACTTGAGGAGAAGTGGCTGCTGCTGCTGCTGAGT	1530
481	GlyArgSerGluThrGluMetLeuGlnLysIleValSerLeuGlnLysValLeuGluGluLysValAlaAlaAlaLeuValSerGln	510
1531	← GTTCTGAGTGGAGCTTTAGAGCTATGTAAGCTCTGTGCAGATAAACAGCAGTTTTCATCAGACCCCGCAAGGACAGAGTACCAGGT	1620
511	ValGlnLeuGluAlaValGlnGluTyrValLysLeuCysAlaAspLysProAlaValSerSerAspProAlaArgThrGluValProGly	540
1621	TTAAGTCAACTGGCAGGAAACACGATGGAGTCCGATGTAATCCGCACTAACCTGGAGAAATCTCAGAGTTAGAAAGCCAGCTGGTGGAAATG	1710
541	LeuSerGlnLeuAlaGlyAsnThrMetGluSerAspValSerAlaLeuThrTrpArgIleSerGluLeuGluSerGlnLeuValGluMet	570
1711	CATTCTAGTTTGAATTTTCAGAGAAAGAGCAAGTAGAAATCCAGAGAAAAATGCTTTTGGAAAAAGAAAGAAATTCGAAGAGCTGCAGAG	1800
571	HisSerSerLeuIleSerGluLysGluGlnValGluIleAlaGluLysAsnAlaLeuGluLysGluLysLysLeuGlnGluLeuGlnLys	1800
1801	CTTGTCAGGACAGTGCAGACAAAGCAGAGAGAGAGAGAGGCAAGCAGGCTTACCGAGATCTTGCGCTTTCGAGTCAACTCAAGAT	1890
601	LeuValGlnAspSerGluThrLysGlnArgGluArgGluArgGlnSerArgLeuHisGlyAspLeuGlyValLeuGluSerThrThrSer	630
1891	GAAGAAAGTGCAGTTTTCGAGAACTTGAAGCTCTTAGAGCTGAGTCAGAGCTCCCAAGGAGAATTCGCAATTACAAGAATTGGCA	1980
631	GluGluSerGlyValPheGlyGluLeuGluAlaLeuArgAlaGluSerAlaAlaProLysGlyGluLeuAlaAsnThrLysGluLeuAla	660
1981	GAAGAACTTCAAGAGAGCTTTTGGTGAAGAAACAAATATGCGATCTTCCAAAGAGTTAAGCCAGTTAGGAGTACAGCTACCGAG	2070
661	GluLysLeuGlnGluGluLeuValLysGluThrAsnMetAlaSerLeuProLysGluLeuSerHisValArgAspGlnLeuThrGlu	690
2071	GCAGAGACAAATTTATCCACTTCTCAGAAAAAGAGACAGAGCTGAGCTACAGAACACCGGACGATCTGCATCTTAGAGCCATGCTCT	2160
691	AlaGluAspLysLeuSerHisPheSerGluLysGluAspLysThrGluValGlnGluHisGlyThrIleCysIleLeuGluProCysPro	720
2161	GGCCAAATAGGTGAAGCTTTTCCCTCCAGACAGAGGGGCTGTCCAAAGTGAACAGCCACCCAGACTCCACAGATCTCTGTTAGAGAT	2250
721	GlyGlnIleGlyGluSerPheAlaSerGlnThrGluGlyAlaValGlnValAsnSerHisThrGlnThrProGlnIleProValArgSer	750
2251	CTGGGAATCAAACCAATTCAGAGAGTACAGTTTCCCGAAGAGTCCGCTGAAATATCAGTGGTTTACTGAAAAAATTGAGCAGATG	2340
751	ValGlyIleGlnThrHisSerGlnSerAspSerSerProGluGluValAlaGluIleIleSerArgPheThrGluLysIleGluGlnMet	780
2341	CGAGAGCTACATGCTGCTGAATTTTGGATATGAGTCCAGACATTTTCAGAAACAGAAACTTTGAAGGGGAACACTGCATTTGCTGTT	2430
781	ArgGluLeuHisAlaAlaGluIleLeuAspMetGluSerArgHisIleSerGluThrGluThrLeuLysArgGluHisCysIleAlaVal	810
2431	CAGTTACTACAGAGAGTGTGCTCTTGAAGTCACTGATACAGGCTTGAAGATGCCAGAGGGTCTCTCAGTTCTCGAATTAACACAT	2520
811	GluLeuLeuThrGluGluCysAlaSerLeuLysSerLeuIleGlnGlyLeuArgMetProGluGlySerSerValProGluLeuThrHis	840
2521	TCTAATGCTTACCAAACTAGAGAAAGTGGGTTCAGTGTATTTGATCAGACTGGGGTCAGGGCACTTTATCTTACAGAGATCAGGATTT	2610
841	SerAsnAlaTyrGlnThrArgGluValGlySerSerAspSerGlySerAspTrpGlyGlnGlyIleTyrLeuThrGlnSerGlnGlyPhe	870
2611	GACACAGCATCAGAGCCCGCAGAGAGAGGTGAACCTTCCAGAGATTCCTTTCCAAAGAAAAAAGGATTACTGAGAGCTGTCCAC	2700
871	AspThrAlaSerGluAlaArgGlyGluGluGlyGluThrSerThrAspSerPheProLysLysIleLysGlyLeuLeuArgAlaValHis	900
2701	AACAGGGCATGCAAGTGTCTTCTCTACCGAGGGTCCCTGTGGCGATGGAGAGGACTATCTGGTCATCAGCTTTCAGAAATCGTGCTG	2790
901	AsnGluGlyMetGlnValLeuSerLeuThrGluGlyProCysGlyAspGlyGluAspTyrProGlyHisGlnLeuSerGluSerTrpLeu	930
2791	GAAGAGAGAGAGCTTACCTCAGTACATCTCATCTCTCAAGGATTTCATTACCAAAATGCAAGTGCAGAGAGAGGTGGAGGTGTATGAC	2880
931	GluGluArgArgAlaTyrLeuSerThrIleSerSerLeuLysAspPheIleThrLysMetGlnValGlnArgGluValGluValTyrAsp	960
2881	AGTTCTCAGTCTCATGAAACATCTCAGACTGGCGAGGTGAACCTCTGCTTGGCTTCAACAAGTTTCTTAAAGGAAACGAGTGTATTA	2970
961	SerSerGlnSerHisGluAsnIleSerAspTrpArgGlyGluLeuLeuAlaLeuGlnGlnValPheLeuArgGluArgSerValLeu	990
2971	← CTAGCTGCACTTAAAGACCGAGCTCAGAGCTTTAGTACTAGAGATGAGCTGGATTATTAACCTGTTTGGAAACCAAGTACCAAGAA	3060
991	LeuAlaAlaPheLysThrGluLeuThrAlaLeuGlyThrArgAspAlaAlaGlyLeuLeuAsnCysLeuGluGlnArgIleProArgThr	1020
3061	GAGTATGA	
1021	GluTyrEnd	
	ATATCAACAGCTATGGAATGTCTACAGAAAGCCGACAGAGGAGTTTGTGTCTGAAATTCAAACATTGCATGCTCAGATGAATGGTAG	
	GAATATGATTCTGAAAGAGAACAGAAAGCAATAAACCAAGCCAGAGCTCTGGAGCACAACCTCAGCAGAGAGAGAGAGAGAGAGAGAGAG	
	CGAGATCGAGGAGCTGGCGAGTACAG	
	CAGAGCGCAATCTGCCAGACCAACTGGAAGTGCAGAGCTGTCGCAAT	

Figure 1 For legend see facing page

microtitre plates were purchased from Xenopore (Saddle Brook, NJ, U.S.A.). Centricon concentrators were obtained from Amicon (Beverly, MA, U.S.A.). PCR Radioactive Labeling

System was purchased from Life Technologies (Gaithersburg, MD, U.S.A.). RNase-protection kits were from Ambion (Austin, TX, U.S.A.). Blocking peptides were synthesized as free acids by

Figure 1 Sequence of AKAP120

(A) The nucleotide sequence (upper line) and deduced amino acid sequence (lower line) from the first in-frame ATG of a cDNA encoding AKAP120. R_{II} B, the R_{II} -binding region is indicated by the underlined region (between the arrows). LZ, three leucine-zipper motifs are indicated by the underlined italics (between the arrows). (B) Diagrammatic representation of the sequencing strategy taken to obtain the entire cDNA sequence of AKAP120.

the University of Georgia (Athens, GA, U.S.A.) Peptide Synthesis Core Laboratory and subsequently reconstituted in distilled water. All other reagents were from standard suppliers and were of the highest purity available.

Phosphorylation of R_{II}

32 P-labelled R_{II} was used for the overlay and binding assay procedures. Recombinant R_{II} produced in BL21(DE3)pLysS bacteria possessing an R_{II} -pET11d expression plasmid (a gift from Dr. John Scott, Vollum Institute, Portland, OR, U.S.A.) was purified by cAMP-affinity chromatography. Recombinant R_{II} was phosphorylated by incubation with bovine A-Kinase catalytic subunit and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as described previously [9].

Cloning of parietal cell AKAP cDNAs

A rabbit parietal cell λ ZAPII cDNA library was prepared with mRNA from > 95% pure rabbit parietal cells. One million plaques were screened in an expression mode (IPTG) using a modified ^{32}P R_{II} overlay procedure (see below). A single positive plaque was identified, isolated and purified through three rounds of dilutional subcloning. Both strands were sequenced by primer walking and restriction fragment subcloning using the DNA fmol sequencing kit dideoxy chain termination method [10]. Sequenase was used with ITP to resolve any compressions.

R_{II} overlay procedure

Nitrocellulose (0.22 μm) filter lifts from the library screening procedure or electroblots were blocked with 5% non-fat dry milk, 0.1% BSA in 25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.05% NaN_3 (Blotto) for 16–24 h at 4 °C before the addition of ^{32}P R_{II} (100 000 c.p.m./ml Blotto, supplemented with 5 mM benzamidine) for 4 h at room temperature. After incubation, the blot was washed five times for 15 min each with the Blotto plus benzamidine, and once briefly with 25 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 0.05% NaN_3 , air-dried and exposed to either XRP-1 film (Kodak) overnight at –70 °C or a PhosphorImaging screen (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Expression of AKAP120 fragment polypeptides

Fragments of AKAP120 were amplified by PCR using Pfu polymerase and primers that contained an *Nde*I restriction site engineered into the sense primer and a *Bam*HI restriction site engineered into the antisense primer. After amplification, the resulting PCR products were subjected to phenol/chloroform

extraction and subsequent restriction digestion. The digested fragments were ligated into the bacterial expression vector pET19b (Novagen) to facilitate the fusion of an N-terminal 10-histidine residue tag to the recombinant peptide.

Expression of recombinant protein was performed by growing BL21(DE3)pLysS transformants to $A_{600} = 0.6$ at 37 °C, inducing expression with 1 mM IPTG for 30 min at either room temperature or 30 °C, and then inhibiting endogenous prokaryotic RNA polymerase with rifampicin (200 $\mu\text{g}/\text{ml}$ final) for 2 additional hours at either room temperature or 30 °C. At the end of the incubation period, recombinant protein was purified over a Ni^{2+} -chelate column (HIS-bind, Novagen) under denaturing conditions (6 M urea) according to the guidelines of the pET system manufacturer (Novagen). The recombinant protein was dialysed extensively against decreasing concentrations of urea and imidazole in 25 mM Hepes, pH 7.0, and the recombinant protein was subsequently concentrated using ultrafiltration (Centricon; Amicon).

SDS/PAGE analysis

Sample extracts from bacterial lysates or purified recombinant protein were resolved by SDS/PAGE (8% gels) and either stained with Coomassie Blue or transferred overnight at 100 mA to nitrocellulose (0.22 μm) for subsequent ^{32}P R_{II} overlay (as described above).

R_{II} -binding assay

R_{II} binding was assessed in recombinant AKAPs bound to Ni^{2+} -chelate microtitre plates. To coat plates, 50 $\mu\text{l}/\text{well}$ 1 $\mu\text{g}/\text{ml}$ histidine-tagged recombinant AKAPs in buffer A (10 mM Tris/HCl, pH 7.5, 50 mM NaCl) were incubated in the Ni^{2+} -chelate microtitre plates at room temperature for 2 h. After being washed three times with buffer A, the plates were blocked with Blotto for 60 min. The wells were incubated for 2 h at room temperature with ^{32}P R_{II} (50 000 c.p.m./well) in 100 μl of Blotto (plus 5 mM benzamidine) in either the absence or presence of various concentrations of unlabelled R_{II} . For experiments involving the use of synthetic blocking peptides, ^{32}P R_{II} was preincubated with various concentrations of peptide for 15 min at room temperature before addition to the plate. A peptide derived from the helical calmodulin-binding site of calmodulin-dependent protein kinase II (MHRQETVDCLKKFNARRKLKGA) was used as a non-specific control peptide. The plates were then washed four times with Blotto, and the adherent counts were determined by liquid-scintillation counting in a Microbeta microtitre plate scintillation counter (Wallac). All determinations were performed in triplicate.

Northern-blot analysis

Polyadenylated RNA was prepared from > 95 % pure parietal and chief cells using the PolyATtract system. mRNA (5 µg) from both parietal and chief cells was resolved on 1 % agarose/formaldehyde gels and transferred to Magnagraph (MSI, Westboro, MA, U.S.A.). An 1100 nt probe corresponding to amino acids 672–1022 in AKAP120 was labelled by PCR amplification in the presence of [α - 32 P]CTP (PCR Radioactive Labeling System). Blots were probed overnight at 42 °C and then washed to high stringency [$0.1 \times$ SSC (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate); 65 °C]. Dried blots were exposed to film for 14 days at -70 °C with two intensifying screens (Reflection, NEN, Boston, MA, U.S.A.).

RNase-protection assay

A 220 nt piece of AKAP120 (nt 2018–2238) was amplified by PCR and cloned into PCRscript. Insert sequence and orientation were verified by double-stranded sequencing. Antisense cRNA transcripts for AKAP120 and glyceraldehyde-3-phosphate dehydrogenase were synthesized using T7 RNA polymerase and [α - 32 P]UTP (Maxiscript; Ambion). The labelled probe (50000 c.p.m.) was then hybridized in solution with 1 µg of total RNA from various rabbit tissues overnight at 42 °C (RNAPII; Ambion). Unprotected RNA fragments were then digested with a mixture of RNase A and T1. Protected fragments were resolved on a urea/6 % polyacrylamide gel and subsequently visualized by exposure to a PhosphorImaging screen or X-ray film.

RESULTS

cDNA cloning of AKAP120

We have screened a λ ZAPII cDNA library, prepared from > 95 % pure rabbit gastric parietal cells, in an expression mode using a modified [32 P] R_{II} electroblot overlay procedure. A single positive plaque was identified, purified and subcloned. The isolated cDNA insert was 3.5 kb in length and coded for a recombinant expressed protein migrating with an apparent molecular mass of 120 kDa. Therefore we have tentatively named this clone AKAP120. Sequence analysis revealed an open reading frame of 3066 nucleotides, 1022 amino acids from the first in-frame ATG (Figure 1A). This protein has a predicted molecular mass of 116.9 kDa and pI of 4.62. In Figure 1(A), the putative α -helical R_{II} -binding region ($R_{II}B$) is indicated by the underline (between arrows), and a region of three predicted leucine-zipper (LZ) motifs resides between residues 977 and 998 (underlined italics, between arrows, Figure 1A), just 24 amino acids from the termination codon. Further sequence analysis indicated a single, but weak, putative A-Kinase phosphorylation site at amino acid 192 (KKXS). In addition, 31 casein kinase II and 14 protein kinase C consensus phosphorylation sites are distributed throughout the molecule. The nucleotide sequence was determined in both directions through restriction fragment subcloning and primer walking. Figure 1(B) illustrates diagrammatically the sequence strategy taken to generate these data.

Sequence identity between this AKAP and other known AKAPs is very low, as has been demonstrated for other AKAPs. However, a significant degree of identity does exist between the area spanning the putative R_{II} -binding region of AKAP120 and the R_{II} -binding region of two other AKAPs, AKAP95 (39 %) and microtubule-associated protein-2 (MAP-2) (44 %) (Figure 2A). Helical-wheel analysis of the putative R_{II} -binding region of AKAP120 illustrated that this sequence represented an α -helical

A.

AKAP120	LEEKVAAALVSQVQLEAV
AKAP95	TPEEVAAEVLAEVITAAV
MAP-2	TAEEVSARIVQVVTAAEV

B.

Figure 2 Sequence analysis of AKAP120

(A) Alignment of the amino acid sequences from the regions spanning the putative R_{II} -binding regions of AKAP120, AKAP95 and MAP-2. (B) Helical-wheel analysis of the R_{II} -binding region of AKAP120, drawn as an α -helix with 3.6 amino acids per turn. The solid area indicates the major hydrophobic face of the helix. The arrow indicates the direction of the helix.

structure with a prominent hydrophobic face (shaded region in Figure 2B). In addition, it should be noted that the opposite face also has significant hydrophobicity.

Mapping the R_{II} -binding region of AKAP120

To further localize the R_{II} -binding region of AKAP120, we constructed recombinant fragments of AKAP120, through PCR amplification, cloning into pET19b, expression in BL21(DE3)pLysS bacteria and subsequent purification of the histidine-tagged recombinant proteins. Truncations were performed at positions of normally occurring methionines. The strategy we took enabled us to synthesize three recombinant fragments of AKAP120 which contained the sequence of amino acids encompassing the putative R_{II} -binding region. The two smallest fragments [AKAP120-(549–1022), AKAP120-(674–1022)] did not contain the putative R_{II} -binding region. Figure 3(A) illustrates a schematic representation of the recombinant truncated fragments that were isolated. After expression, the recombinant fragments were purified to homogeneity and subjected to SDS/PAGE analysis. A representative Coomassie-stained SDS/polyacrylamide gel illustrating the recombinant proteins is shown in Figure 3(B), with a corresponding [32 P] R_{II} overlay depicted in Figure 3(C). As hypothesized, the three larger recombinant fragments of AKAP120 [AKAP120-(183–1022), AKAP120-(287–1022) and AKAP120-(489–1022)] bound [32 P] R_{II} , whereas AKAP120-(549–1022) and AKAP120-(674–1022) did not (Figure 3C). These results localize the R_{II} -binding region of AKAP120 to the area between amino acids 489 and 549. This stretch of amino acids contains the putative α -helical R_{II} -binding region between amino acids 503 and 516.

Figure 3 Mapping the R_{II} -binding region of AKAP120

(A) Schematic representation of the recombinant AKAP120 fragments synthesized for R_{II} -binding analysis. (B) Coomassie-stained SDS/polyacrylamide gel of the recombinant AKAP120 fragments. (C) [32 P] R_{II} blot overlay demonstrating that only the three larger recombinant AKAP120 fragments bound R_{II} .

Figure 4 R_{II} -binding affinity assay on recombinant fragments of AKAP120

R_{II} binding to the recombinant AKAP120 fragments that demonstrated R_{II} binding [●, AKAP120-(183–1022); ■, AKAP120-(287–1022); ▲, AKAP120-(489–1022)] was performed using a modified [32 P] R_{II} overlay procedure. The points are means $\pm$ S.E.M. for three experiments each performed in triplicate.

Figure 5 Peptide inhibition of R_{II} binding to AKAP120

R_{II} binding to the recombinant fragment AKAP120-(287–1022) was performed using [32 P] R_{II} preincubated with increasing concentrations of synthetic peptides spanning the α -helical R_{II} -binding region of the AKAP, HT-31 (●) or AKAP120 (■). Points are means $\pm$ S.E.M. for two experiments each performed in triplicate.

Figure 6 Disruption of α -helix abolishes peptide inhibition

Strips of electroblots containing approx. 100 ng of AKAP120-(287–1022) were probed with [32 P] R_{II} in the absence of added peptide (lane A), in the presence of 1 μ M AKAP120-(489–522) (AKAPep) (lane B) or in the presence of 1 μ M AKAP120-(489–522) containing a leucine to proline substitution at position 507 [AKAPep(L > P)] (lane C).

R_{II} -binding affinity studies

A high-affinity protein–protein interaction (K_{app} in the nanomolar range) usually exists between AKAPs and R_{II} (reviewed in [1]). Therefore we sought to determine the nature of the interaction between AKAP120 and R_{II} . Using the recombinant fragments of AKAP120 that demonstrated R_{II} binding (Figure 3C), we developed a novel binding-affinity assay utilizing the histidine tag on the recombinant proteins and Ni^{2+} -chelate microtitre plates to evaluate [32 P] R_{II} -binding. This assay enabled us to determine actual solution binding affinities as compared with affinity determination by probing denatured electroblots with [32 P] R_{II} . Unlabelled R_{II} inhibited [32 P] R_{II} binding to the recombinant proteins in a dose-dependent manner (Figure 4). The results demonstrate that there is a high-affinity interaction between AKAP120 and R_{II} , with a K_{app} ranging between 50 and 120 nM for the three recombinant fragments tested. No binding

Figure 7 Distribution of AKAP120 mRNA

(A) Northern-blot analysis of AKAP120; 5 μ g of mRNA from parietal cells (P) and chief cells (C) were probed for AKAP120. The locations of 18 S and 28 S rRNA markers are noted on the left. A 3.5–4.0 kb species was observed in parietal cells, but not in chief cells. (B) RNase-protection analysis of AKAP120 mRNA. A 220 nt antisense cRNA from AKAP120 was used as a probe to survey rabbit tissues by RNase protection. mRNA was detected in all tissues examined, with an enrichment in fundic and jejunal mucosa. B, brain; F, fundus; P, pancreas; I, ileum; C, colon; J, jejunum. The results of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assays on identical samples are shown for comparison. The results are representative of three separate experiments.

of [32 P]R_{II} to AKAP120-(549–1022) and AKAP120-(674–1022) could be detected using this assay (results not shown), confirming the findings presented in Figure 3(C).

Further determination of the binding affinity between AKAP120 and R_{II} was carried out using the microtitre binding assay and synthetic peptides spanning the R_{II}-binding α -helical region of the AKAPs, HT-31 and AKAP120. Preincubation of [32 P]R_{II} with synthetic peptides containing either the R_{II}-binding region of AKAP120-(489–522) or the R_{II}-binding region of the AKAP, HT-31, inhibited in a dose-dependent manner [32 P]R_{II} binding to recombinant AKAP120-(287–1022) (Figure 5). The HT-31 peptide inhibited [32 P]R_{II} binding with an apparent IC₅₀ of 1 nM, and AKAP120 peptide demonstrated an apparent IC₅₀ of 20 nM. Both peptides could completely inhibit [32 P]R_{II} binding. A representative blot overlay illustrating inhibition of the binding of [32 P]R_{II} to AKAP120-(287–1022) is shown in Figure 6. In addition, both peptides inhibited R_{II} binding to rabbit brain AKAPs in blot overlays (results not shown). A synthetic peptide containing a leucine to proline substitution at amino acid 507, disrupting the α -helix, failed to inhibit [32 P]R_{II} binding at concentrations up to 100 μ M (Figure 6). In addition, use of a non-specific peptide of similar length and pI to the HT-31 peptide had no effect on the binding of [32 P]R_{II} to any of the recombinant AKAP120 fragments which demonstrated positive binding (results not shown).

Distribution of AKAP120 mRNA

Northern-blot analysis demonstrated only faint bands of a 3.5–4.0 kb mRNA species in the polyadenylated RNA isolated from purified parietal cells, whereas no message was detected in purified chief cells (Figure 7A). To assess further the tissue distribution of AKAP120, we utilized an RNase-protection assay to survey a variety of tissues for the presence of AKAP120 mRNA. A 220 nt fragment of AKAP120 was synthesized and

utilized to produce antisense cRNA probes. AKAP120 mRNA was detected in all tissues examined, with relative enrichment in the gastric fundic and jejunal mucosa (Figure 7B).

DISCUSSION

We have described the identification and characterization of a novel AKAP isolated from a rabbit gastric parietal cell cDNA library. The expressed recombinant protein migrated with an apparent molecular mass of 120 kDa; therefore we have tentatively named this clone AKAP120. Sequence analysis has revealed a putative R_{II}-binding region between amino acids 503 and 516. This region has significant similarity to the areas spanning the R_{II}-binding regions contained in two other AKAPs, AKAP95 and MAP-2. Helical-wheel analysis of the putative R_{II}-binding region of AKAP120 (amino acids 503–516) revealed that this sequence represents an α -helical structure containing several long-chain aliphatic residues. AKAP120 also contains a series of three predicted leucine-zipper motifs at its C-terminus, between amino acids 977 and 998. Leucine-zipper motifs mediate both protein dimerization and DNA binding [11,12]. Recently, Rubin and colleagues [13] have characterized a novel AKAP, S-AKAP84, which targets the type-II A-Kinase to the mitochondrial outer membrane. This AKAP, like AKAP120, also contains a series of leucine-zipper motifs. However, it is not known whether these motifs participate in the dimerization of S-AKAP84 molecules. The role for the series of leucine zippers in the interaction of AKAP120 with itself or with nucleic acid binding is unknown at this time. However, we have been unable to demonstrate any binding of AKAP120 to DNA in gel mobility-shift assays (J. R. Goldenring and D. T. Dransfield, unpublished work). Therefore AKAP120 probably does not participate in DNA binding.

The AKAP120 sequence also contains a series of consensus phosphorylation sites. A single putative, but weak, A-Kinase phosphorylation site is present at amino acid 192 (KKXS), and 31 casein kinase II and 14 protein kinase C consensus phosphorylation sites are distributed throughout the molecule. Many AKAPs cloned to date are substrates for A-Kinase, and Scott and colleagues [14] recently demonstrated that AKAP79 could be phosphorylated by protein kinase C.

Database searches for sequence similarities between AKAP120 and other proteins revealed a 10–15% similarity to myosin(s), suggesting a coiled-coil tertiary structure towards the C-terminal half of AKAP120. These data point to a possible role for AKAP120 as a projection protein, similar to that suggested for MAP-2. In addition, we have detected a doublet of acidic R_{II}-binding proteins (~130–140 kDa) in the Triton X-100-insoluble fraction of purified gastric parietal cells (D. T. Dransfield and J. R. Goldenring, unpublished work), indicating a potential cytoskeletal association. The determination of the specific subcellular localization of AKAP120 in the gastric parietal cell awaits the development of suitable monoclonal antibodies.

We have developed a novel affinity-binding assay utilizing the histidine-tagged recombinant AKAP120 fragments to obtain the K_{app} between AKAP120 and R_{II}. This method has allowed us to determine solution affinity-binding constants without the denaturation that is part of the [32 P]R_{II} blot overlay procedure. All three recombinant AKAP120 fragments containing the putative R_{II}-binding region bound [32 P]R_{II}, and this binding was competitive with excess quantities of unlabelled R_{II}. The results from these affinity-binding experiments demonstrated that a high-affinity protein–protein interaction takes place between R_{II} and AKAP120, with a K_{app} between 50 and 120 nM for the three recombinant AKAP120 fragments tested. Complementary ex-

periments were conducted using this assay to determine the binding affinity for the synthetic peptides that span the area of the putative R_{II} -binding regions of either AKAP120 or the AKAP, HT-31. The peptide spanning the R_{II} -binding region of HT-31 has been used to block R_{II} from binding to all known AKAPs [4]. The data presented in Figure 5 illustrate that the peptides spanning the R_{II} -binding regions of either AKAP120 or HT-31 dose-dependently inhibited the binding of [32 P] R_{II} to recombinant AKAP120-(287–1022). The IC_{50} for these peptides was in a similar range to that obtained using unlabelled R_{II} to block the binding of [32 P] R_{II} to the recombinant AKAP120 fragments (Figure 4). Substitution of a proline for a leucine at position 507, disrupting the α -helix, abolished peptide inhibition of R_{II} binding to AKAP120-(287–1022). These findings confirm the studies of Scott and colleagues [15,16], which demonstrated that insertion of prolines into the R_{II} -binding helical region completely eliminated the interaction between AKAPs and R_{II} .

Taken together, these results suggest that an α -helical region is required for the interaction between AKAP120 and R_{II} , and confirm the findings using the recombinant AKAP120 fragments which demonstrated a high-affinity protein–protein interaction between R_{II} and AKAP120. However, although the putative R_{II} -binding helical region does possess a hydrophobic face (shaded region in Figure 2B), the opposite face also displays significant hydrophobicity. Thus this structure does not adhere strictly to the proposed amphipathic α -helical structure of R_{II} -binding regions [4]. In addition, Rubin and colleagues [17] have previously demonstrated the importance of long-chain aliphatic amino acids in R_{II} binding to AKAP75. The presence of three long-chain aliphatic residues in the major hydrophobic face of the putative R_{II} -binding α -helix in AKAP120 suggests that such long-chain aliphatic side chains are of primary importance for the interaction between R_{II} and this AKAP.

In gastric parietal cells, histamine, through activation of adenylate cyclase, elicits the phosphorylation of several proteins including ezrin [18–20]. In addition, stimulation of gastric parietal cells with cAMP-elevating agents elicits dephosphorylation of particulate-associated R_{II} [6]. The rapid dephosphorylation (< 1 min) suggests that the type-II A-Kinase may be closely associated with a phosphoprotein phosphatase. The data from previous studies in our laboratory indicate that a membrane-bound kinase–phosphatase system may be present in gastric parietal cell membranes [6]. AKAP120 may be responsible for site-specific assembly of such multienzyme complexes with their substrates in gastric parietal cells. Indeed, AKAP79 is associated with protein phosphatase 2b and protein kinase $C\alpha$ in the brain [14,21].

We have previously reported the identification of an apparent parietal cell-specific AKAP (AKAP78), which is localized to the Triton X-100-insoluble fraction of gastric parietal cells [8].

Recent experimental findings in our laboratory have identified AKAP78 as ezrin, the canalicular-associated actin-binding protein [22]. On the basis of the findings presented in this study, it appears that AKAP120 is a new member of a growing family of signal-transduction molecules which anchor the type-II A-Kinase holoenzyme to specific subcellular domains. Northern blot and RNase-protection analysis revealed that the AKAP120 mRNA is widely distributed among the tissues examined, with enrichment in the gastric fundic and jejunal mucosa, specifically in gastric parietal rather than chief cells. Subcellular localization of A-Kinase in association with its substrates may lead to co-ordinated phosphorylation events in the gastric parietal cell.

We thank Dr. John Scott for the gift of the murine R_{II} cDNA in pET11d. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) grants DK48370 and DK43405 and a Veterans Affairs Merit Review Award to J.R.G. D.T.D. was the recipient of a National Research Service Award DK09026.

REFERENCES

- 1 Scott, J. D. and McCartney, S. (1994) *Mol. Endocrinol.* **8**, 5–11
- 2 Scott, J. D. and Carr, D. W. (1992) *News Pharmacol Sci* **7**, 143–148
- 3 Hirsch, A. H., Glantz, S. B., Li, Y., You, Y. and Rubin, C. S. (1992) *J. Biol. Chem.* **267**, 2131–2134
- 4 Carr, D. W., Hausken, Z. E., Fraser, I. D. C., Stofko-Hahn, R. E. and Scott, J. D. (1992) *J. Biol. Chem.* **267**, 13376–13382
- 5 Chew, C. S. (1985) *J. Biol. Chem.* **260**, 7540–7550
- 6 Goldenring, J. R., Asher, V. A., Barreuther, M. F., Lewis, J. J., Lohmann, S. M., Walter, U. and Modlin, I. M. (1992) *Am. J. Physiol.* **262**, G763–G773
- 7 Gjertsen, B. J., Fauske, B. and Doskeland, S. O. (1993) *Biochem. J.* **251**, 7526–7529
- 8 Dransfield, D. T., Bradford, A. J. and Goldenring, J. R. (1995) *Biochim. Biophys. Acta* **1269**, 215–220
- 9 Scott, J. D., Stofko, R. E., McDonald, J. R., Comer, J. D., Vitalis, E. A. and Mangili, J. (1990) *J. Biol. Chem.* **265**, 21561–21566
- 10 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 11 McKnight, S. L. (1991) *Sci. Am.* **264**, 54–64
- 12 O Shea, E., Rutkowski, R. and Kim, P. S. (1989) *Science* **243**, 538–542
- 13 Lin, R.-Y., Moss, S. B. and Rubin, C. S. (1995) *J. Biol. Chem.* **270**, 27804–27811
- 14 Coghlan, V. M., Perrino, B. A., Howard, M., Langeberg, L. K., Hicks, J. B., Gallatin, W. M. and Scott, J. D. (1995) *Science* **267**, 108–111
- 15 Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Cone, R. D. and Scott, J. D. (1992) *J. Biol. Chem.* **267**, 16816–16823
- 16 Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D. C., Bishop, S. M., Acott, T. S., Brennan, R. G. and Scott, J. D. (1991) *J. Biol. Chem.* **266**, 14188–14192
- 17 Glantz, S. B., Li, Y. and Rubin, C. S. (1993) *J. Biol. Chem.* **268**, 12796–12804
- 18 Chew, C. S. and Brown, M. R. (1987) *Am. J. Physiol.* **253**, G823–G829
- 19 Urushidani, T., Hanzel, D. K. and Forte, J. G. (1989) *Am. J. Physiol.* **256**, G1070–G1081
- 20 Oddsdottir, M., Goldenring, J. R., Adrian, T. E., Zdon, M. J., Zucker, K. A. and Modlin, I. M. (1988) *Biochem. Biophys. Res. Commun.* **154**, 489–496
- 21 Klauk, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S. and Scott, J. D. (1996) *Science* **271**, 1589–1592
- 22 Goldenring, J. R., Dransfield, D. T., Bradford, A. J., Smith, J., Martin, M. and Mangeat, P. H. (1997) *EMBO J.*, in the press