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Purification of an angiotensin II binding protein by using antibodies to a peptide encoded by angiotensin II complementary RNA

(angiotensin II receptor/complementary peptides/molecular recognition code/immunoaffinity chromatography)

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ABSTRACT **We have generated a monospecific antibody to a synthetic peptide encoded by an RNA complementary to the mRNA for angiotensin II (AII) and determined whether this antibody recognizes the AII receptor. We demonstrate that the antibody competes specifically with ^{125}I-labeled AII for the same binding site on rat adrenal membranes. Furthermore, we show that this antibody inhibits the secretion of aldosterone from cultured rat adrenal cells, suggesting that the antibody recognizes the biologically relevant AII receptor. Finally, we demonstrate that antibody to the complementary peptide can be used to immunoaffinity-purify a protein of M_r 66,000 that specifically binds radiolabeled AII.**

Angiotensin II (AII), an octapeptide hormone, is the biologically active component of the renin–angiotensin system (1, 2). AII mediates vasoconstriction (3) and aldosterone secretion (4) through specific interactions with AII receptors present on vascular smooth muscle (3) and adrenal glands (4), respectively. To understand how the binding of AII with its specific receptor mediates a precise biochemical effect, the AII receptor must be purified and characterized. Recently, several research groups have identified an apparent AII binding protein of M_r 66,000 by means of photoaffinity labeling (5–9), crosslinking (10, 11), and affinity chromatography (12). While these techniques are useful tools for the characterization of the AII receptor, they have not resulted in its purification to homogeneity.

Recently, it was hypothesized that there is a molecular recognition code in which peptide ligands and their receptor binding sites can be encoded by complementary nucleotide sequences (13). In support of this hypothesis, several laboratories have demonstrated that peptides encoded by complementary strands of nucleic acids (designated "complementary peptides") have the ability to bind one another (13–15) using the same reading frame. For example, it was shown that complementary peptides to corticotropin, γ-endorphin, and luteinizing hormone-releasing hormone would bind to their respective hormone with high affinity and specificity, suggesting that the complementary peptides resembled receptor binding sites (13, 16–20). Furthermore, it was demonstrated that antibodies directed against a peptide complementary to a particular hormone would also bind to that hormone's receptor binding site (13, 16–20). In fact, antibodies directed against peptides complementary to corticotropin, γ-endorphin, and luteinizing hormone-releasing hormone have been used to immunoaffinity-purify these specific hormone receptors (13, 16–20).

In this report, we have investigated the usefulness of the molecular recognition hypothesis in the purification of the AII receptor. Specifically, we have determined whether a peptide, IIA, specified by RNA complementary to the mRNA of AII would bind AII and, if so, whether antibodies to such a peptide would sufficiently resemble an AII binding site that they would crossreact with the AII receptor.

MATERIALS AND METHODS

Materials. Synthetic [Asp^1,Ile^5]AII, [Sar^1,Ile^8]AII (Sar = sarcosine), and 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate (CHAPS) were purchased from Sigma. ^{125}I-labeled AII (^{125}I-AII; ≈1880 μCi/μg; 1 Ci = 37 GBq) was from New England Nuclear. Disuccinimidyl suberate and Reacti-Gel 6X were from Pierce. Affi-Gel 10 and standard molecular weight markers were Bio-Rad products. An aldosterone radioimmunoassay kit was obtained from Diagnostic Products (Los Angeles).

Peptides and Antiserum. The octapeptide (IIA) specified by the RNA sequence complementary to the mRNA sequence for rat [Asp^1,Ile^5]AII (21) was synthesized (Triton Biosciences, Alameda, CA) by standard solid-phase methods and purified (>97%) by HPLC. Control peptide (Ala-Ser-Thr-Thr-Thr-Asn-Tyr-Thr-COOH) was also synthesized and purified as above. Rabbit antiserum against complementary peptide IIA was prepared and purified as reported (13). Anti-IIA IgG was purified by affinity chromatography. Briefly, the IIA peptide was coupled to an agarose gel bead support (Affi-Gel 10) as described by the manufacturer. DEAE-purified IgG or serum from rabbits that had been immunized with complementary peptide IIA was then chromatographed on the IIA-Affi-Gel 10 affinity column. Antibodies (anti-IIA IgG) that bound to the column were eluted with 3 M $MgCl_2$ and collected. The eluted material was then dialyzed against phosphate-buffered saline (PBS) and dehydrated by extraction with polyethylene glycol to yield a protein concentration of 3 mg/ml.

Aldosterone Assay. Collagenase-dispersed glomerulosa cells were prepared from rat adrenal glands as described (22). Adrenal cells were resuspended in medium 199 containing 0.2% bovine serum albumin. Cell viability as monitored by trypan blue exclusion was usually >90%. One-milliliter aliquots of the cell suspension (≈10^5 cells) were incubated with AII, anti-IIA IgG, preimmune IgG, or AII with anti-IIA IgG for 2 hr at 37°C under 5% CO_2/95% air. The cells were then pelleted, and the aldosterone content of the media was measured by direct radioimmunoassay (23).

Preparation of Rat Adrenal Membranes. Crude rat membrane preparations were obtained as described (24) with a

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Abbreviations: AII, angiotensin II; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propanesulfonate; IIA, a peptide synthesized according to the RNA sequence complementary to the mRNA of rat AII; Sar, sarcosine.

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		1			4			8	
AII		Asp	Arg	Val	Tyr	Ile	His	Pro	Phe
mRNA	5'	GAC	CGC	GUA	UAC	AUC	CAC	CCC	UUU 3'
Complementary RNA	3'	CUG	GCG	CAU	AUG	UAG	GUG	GGG	AAA 5'
Complementary Peptide IIA		Val	Ala	Tyr	Val	Asp	Val	Gly	Lys
		8			4			1	

FIG. 1. Amino acid sequence of the complementary octapeptide IIA encoded by an RNA that is complementary to the mRNA for AII. The amino acid and nucleotide sequence for rat AII were from Ohkubo *et al.* (21). Codons in the complementary RNA were assigned in the 5' → 3' direction to derive the amino acid sequence of the complementary peptide IIA.

few minor modifications. Briefly, fresh rat adrenal glands were homogenized in ice-cold 50 mM Tris·HCl, pH 7.5/5 mM EDTA/0.1 mM phenyl methylsulfonyl fluoride with a loose-fitting Dounce homogenizer. The homogenate was then filtered through nylon gauze and centrifuged at 600 × *g* for 10 min. The supernatant was centrifuged at 30,000 × *g* for 30 min, and the pellet (adrenal particles) was resuspended in the appropriate buffer. In some experiments, the adrenal particles were purified further with the use of discontinuous sucrose gradients (22).

AII Receptor Binding Assays. ^{125}I-AII (0.01 nM) was incubated for 60 min at 22°C in 0.01–0.2 ml of assay buffer (50 mM Tris·HCl, pH 7.5/120 mM NaCl/5 mM EDTA/0.2% bovine serum albumin) with 100–200 μg of adrenal particles and various concentrations of unlabeled AII, [Sar^1,Ile^8]AII, control peptide, anti-IIA IgG, or control IgG. The reactions were stopped by filtration on GF/B glass filters, followed by four rapid washings with 5 ml of ice-cold incubation buffer. Nonspecific binding was measured in the presence of an excess of unlabeled AII (10 μM) and always represented <10% of total bound radioactivity.

AII Receptor Affinity Purification. Affinity-purified anti-IIA IgG was coupled to agarose beads, Reacti-Gel 6X, as described by the manufacturer. Rat adrenal particles (5 mg) were resuspended in PBS and solubilized with CHAPS as described (17). The solubilized adrenal particles were centrifuged at 700 × *g* for 10 min, and the supernatant was diluted 1:6 with PBS. The diluted supernatant was then chromatographed on the anti-IIA IgG affinity column. Material that bound to the column was eluted with 3 M $MgCl_2$, collected, and dialyzed against H_2O overnight. The material was then dehydrated by extraction with polyethylene glycol and subsequently analyzed by polyacrylamide gel electrophoresis. In some experiments, adrenal particles were resuspended in PBS, sonicated for 1 min, and then subjected to affinity chromatography.

Specific AII Binding to Immunoaffinity-Purified Protein. Protein that bound to the anti-IIA IgG affinity column (1–50 μg) was incubated with ^{125}I-AII (0.2 nM) in the presence or absence of nonlabeled AII (10 μM) at 4°C for 1 hr in 500 μl of PBS (pH 7.4). These samples were then crosslinked with 3.0 mM disuccinimidyl suberate in dimethyl sulfoxide for 15 min at 25°C. Finally, the samples were dialyzed for 18 hr against PBS to remove free ^{125}I-AII, hydroextracted with polyethylene glycol, and subjected to polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed on homogeneous slab gels (7.5% acrylamide) (25). After electrophoresis, gels were stained for protein by a silver-staining method as described (26). After staining, some gels were dried and subjected to autoradiography at −90°C. The autoradiograph was scanned with a densitometric scanner (Hoefer, San Francisco).

RESULTS

We initially investigated whether a complementary AII peptide would interact with AII in a specific manner. Therefore, we synthesized a complementary AII peptide based on the rat angiotensinogen cDNA nucleotide sequence obtained from Ohkubo *et al.* (21). The amino acid and mRNA sequences for rat AII are shown in Fig. 1. Also shown in Fig. 1 is the nucleotide sequence of the complementary RNA and the amino acid sequence of the complementary peptide (IIA) when assigned in the 5' → 3' direction and in the same reading frame. The complementary peptide IIA (Fig. 1, Lys-Gly-Val-Asp-Val-Tyr-Ala-Val-COOH) was then tested for the ability to specifically inhibit the binding of ^{125}I-AII with AII receptors present on rat adrenal particles or purified rat adrenal plasma membranes (see *Materials and Methods* and ref. 27). Displayed in Fig. 2 is a typical ^{125}I-AII competition binding experiment using rat adrenal particles. The results show that IIA is capable of inhibiting the binding of ^{125}I-AII with rat adrenal AII receptors in a manner comparable to [Sar^1,Ile^8]AII and nonlabeled AII (similar results were also obtained with purified rat adrenal plasma membranes). The inhibition was dose dependent (Fig. 2) and did not occur if the cells were preincubated with IIA and subsequently washed (data not shown), indicating that IIA is not binding to the AII receptor. Furthermore, the inhibition of ^{125}I-AII binding appeared to be specific because control peptides did not significantly inhibit the binding of radiolabeled AII, even at concentrations of 0.1 mM (Fig. 2). These experiments suggest that the complementary peptide IIA

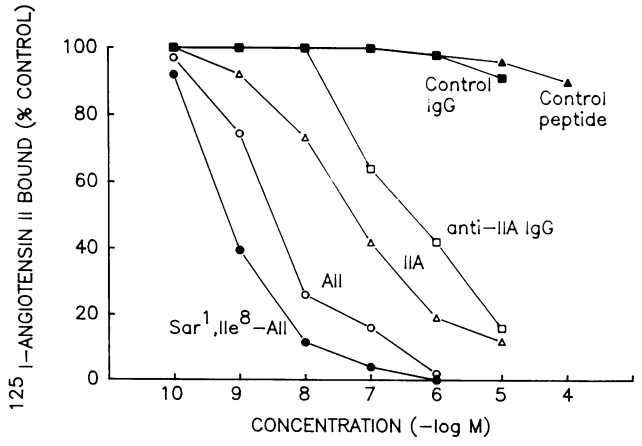

FIG. 2. Competition curves for ^{125}I-AII binding to rat adrenal particles. ^{125}I-AII (0.01 nM) was incubated with 100–200 μg of rat adrenal particles and increasing concentrations of unlabeled ligands for 60 min at 25°C. The amount of bound ^{125}I-AII was determined by filtration as described. The ligands used were [Sar^1,Ile^8]AII (●), AII (○), complementary peptide IIA (△), anti-IIA IgG (□), control IgG (■), and control peptide (▲).

interacts specifically with ^{125}I-AII and, thus, interferes with its ability to bind to adrenal AII receptors.

To determine if the interaction between IIA and AII was due to the complementary peptide having a shape or conformation resembling the AII receptor, an antibody was prepared against IIA and evaluated for its ability to recognize the AII receptor binding site. Fig. 2 shows that affinity-purified anti-IIA IgG inhibits the binding of radiolabeled AII to AII receptors. Additionally, Table 1 shows that anti-IIA IgG inhibits the secretion of aldosterone from freshly dispersed rat adrenal glomerulosa cells. In contrast, control IgG (preimmune serum) did not block ^{125}I-AII binding or inhibit the spontaneous or AII-mediated release of aldosterone (data not shown). Taken together, these results suggest that anti-IIA IgG specifically recognizes AII binding sites.

To investigate whether antibodies against IIA could be used to affinity-purify the AII binding protein, anti-IIA IgG was coupled to Reacti-Gel agarose beads. Rat adrenal particles were solubilized with CHAPS and chromatographed on the immunoaffinity column. The material that bound to the column was eluted and subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis. The electrophoretic results presented in Fig. 3 show that the immunoaffinity-purified material consists of a single protein species. Purified protein that was treated with 2-mercaptoethanol (5%) migrated as a M_r 66,000 ± 2,000 polypeptide during electrophoresis (Fig. 3, lane 2). Interestingly, in the absence of a reducing agent, the purified protein migrated faster, with a M_r 58,000 ± 2,000 (Fig. 3, lane 1). These data suggest that the immunoaffinity-purified protein contains intramolecular disulfide bridges.

To demonstrate that this protein actually contains the AII binding site, it was incubated with ^{125}I-AII in the absence or presence of unlabeled AII and crosslinked with disuccinimidyl suberate. The crosslinked material was electrophoresed, silver-stained, and finally subjected to autoradiography. The silver-stained gel displayed in Fig. 4*A* again shows that the immunoaffinity-purified material consists of a single protein species (M_r 66,000 ± 2,000), even with the lanes being overloaded (lanes 1 and 2). Fig. 4*B* shows the autoradiograph of this gel. It is apparent that the immunoaffinity-purified protein could bind ^{125}I-AII (Fig. 4*B*, lane 1). Furthermore, these results strongly suggest that this binding is specific, since the amount of radiolabeled AII binding was reduced when the protein was incubated in the presence of unlabeled AII (Fig. 4*B*, lane 2). In fact, the densitometric evaluation of the data in Fig. 4*B* shows that ^{125}I-AII binding was reduced ≈80% in the presence of unlabeled AII (Fig. 4*C*).

DISCUSSION

Recently, it was hypothesized that there is a molecular recognition code in which peptide ligands and their receptor binding sites can be encoded by complementary nucleotide sequences (13). To test this hypothesis, we have utilized the nucleotide sequence of the rat angiotensinogen cDNA (21) to synthesize a peptide complementary to AII.

In this report, we demonstrate that the complementary peptide IIA specifically inhibits ($K_d \approx 5 \times 10^{-8}$ M) the binding of ^{125}I-AII to AII receptors present on rat adrenal membranes. These results indicate that AII and its complementary peptide IIA will indeed interact with each other, suggesting that IIA does resemble the AII receptor binding site.

If the complementary peptide IIA resembles the AII binding site, then antibodies generated against IIA should

Table 1. Aldosterone production in rat adrenal glomerulosa cells

Stimulus	Aldosterone production, pg/ml (n = 4)
None	330 ± 46
AII (1 μM)	659 ± 28
Anti-IIA IgG (1 μM)	189 ± 26
AII (1 μM) + anti-IIA IgG (1 μM)	199 ± 34

Dispersed rat adrenal glomerulosa cells were suspended in serum-free medium 199 at 1 × 10^5 cells per tube. The cells were incubated for 2 hr at 37°C with AII, control IgG, anti-IIA IgG, or AII and anti-IIA IgG. After incubation, samples were centrifuged, and the cell-free medium was assayed for aldosterone content. Each value represents the mean ± SD of four experiments, each point assayed in duplicate. Control preimmune serum did not inhibit the spontaneous or AII-mediated release of aldosterone.

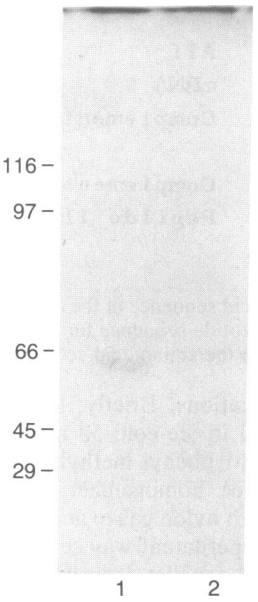

FIG. 3. NaDodSO$_4$/polyacrylamide gel electrophoresis of immunoaffinity-purified material. Rat adrenal particles were solubilized with CHAPS. The solubilized material was chromatographed on an anti-IIA IgG affinity column. Bound material was eluted, electrophoresed on a 7.5% NaDodSO$_4$/polyacrylamide gel, and subsequently silver-stained. Lanes: 1, immunoaffinity-purified material without 2-mercaptoethanol; 2, immunoaffinity-purified material with 2-mercaptoethanol (5%). The molecular weight markers were carbonic anhydrase (M_r, 29,000), ovalbumin (M_r, 45,000), bovine serum albumin (M_r, 66,000), phosphorylase b (M_r, 97,400), and β-galactosidase (M_r, 116,000).

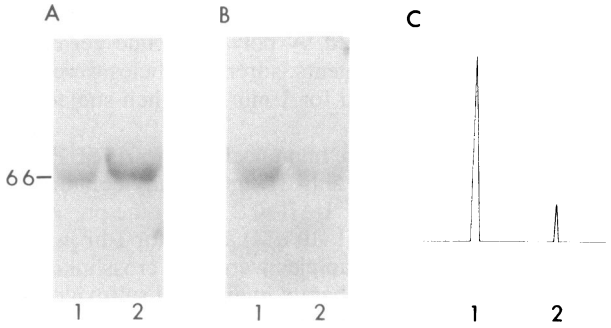

FIG. 4. Specific binding of ^{125}I-AII to immunoaffinity-purified protein. Immunoaffinity-purified material was labeled (^{125}I-AII) in the absence or presence of AII (1 μM). The material was then crosslinked with disuccinimidyl suberate and electrophoresed on a NaDodSO$_4$/7.5% polyacrylamide gel. (*A*) Crosslinked immunoaffinity protein visualized by silver-staining. (*B*) Autoradiograph of the NaDodSO$_4$/polyacrylamide gel in *A*. The autoradiograph was developed after exposure at −80°C for 10 days. (*C*) Densitometric evaluation of the autoradiogram in *B*. Lane 1 is scaled to 100% absorbance. Lanes in *A–C*: 1, protein labeled in the absence of AII; 2, protein labeled in the presence of AII.

crossreact with the AII receptor. Our results show that anti-IIA IgG specifically inhibits the binding ($K_d \approx 4 \times 10^{-7}$ M) of ^{125}I-AII with rat adrenal AII receptors. Additionally, the anti-IIA IgG inhibits aldosterone secretion from freshly dispersed rat adrenal glomerulosa cells. The inhibition of aldosterone production was not caused by toxicity of the antibody as judged by the ability of such cells to exclude trypan blue dye. These results suggest that the complementary peptide antibody is behaving like an AII antagonist. These results also provide strong evidence that antibodies against the complementary peptide IIA bind to the biologically relevant site, or AII receptor, involved in the regulation of aldosterone secretion.

Because anti-IIA IgG appears to recognize the biologically relevant AII binding site, this antibody should be capable of immuno-purifying the molecule. The results show that anti-IIA IgG can purify a single protein species of M_r 66,000 ± 2,000 under reducing conditions. Moreover, the immunoaffinity-purified protein specifically binds ^{125}I-AII, strongly suggesting that the purified material is an AII binding protein.

The molecular weight of the immunoaffinity-purified AII binding protein (66,000) is in excellent agreement with published data obtained by other purification techniques. For example, experiments utilizing photoaffinity-labeled AII analogs have shown that AII binding protein from various mammalian tissues has a M_r of 60,000–68,000 (5–9). Several studies that used crosslinking techniques on solubilized membranes also suggest that the AII binding protein has a M_r of 68,000 (10, 11). Finally, Sen *et al.* (12) have shown by AII affinity purification and immunoprecipitation with anti-AII antibodies that they can purify a putative AII binding protein of M_r 66,000.

Our results show that the immunoaffinity-purified AII binding protein displays an electrophoretic mobility shift after reduction with 2-mercaptoethanol, suggesting the presence of intramolecular disulfide bridge(s) in the protein. Other purified receptors, including β_1- and β_2-adrenergic receptors (28, 29), the hepatic glucagon receptor (30), the opiate receptor purified from bovine striatum (31), the receptors for interleukin 2 (32) and interleukin 3 (33), the luteinizing hormone/human chorionic gonadotropin receptor (34), and the putative AII binding protein (12) display a similar mobility shift after reduction. Moxham and Malbon (28) have shown that the integrity of one or more intramolecular disulfide bridges is essential for ligand binding, and chemical cleavage of these bonds destabilizes the ability of a β_1-adrenergic receptor to bind radioligands. Creighton (35) has suggested that agonist binding may promote disulfide exchange reactions and/or cleavage of intramolecular disulfide bridges and transform the receptor into a structurally altered state capable of activating guanine nucleotide proteins. Recently, it has been shown that AII receptors interact with these regulatory proteins (36–38). Taken together, these results indicate that disulfide bridges present in the AII binding protein may play an important role in transforming the AII receptor into a conformation capable of activating guanine nucleotide binding proteins.

This report describes the purification of an AII binding protein that retains its capacity to specifically bind ^{125}I-AII. Our ability to generate microgram quantities of the purified AII binding protein will undoubtedly facilitate a more detailed biochemical analysis of the AII receptor. Furthermore, these findings provide strong support for the hypothesis that ligands and receptor binding sites can be derived from complementary sequences of nucleic acids.

Note Added in Proof. Close inspection of recently described peptide antagonists of AII revealed that one of these (TBI–31) would result from a 3′-to-5′ reading of RNA complementary to that of AII (39). In this study, other peptides were complementary at the amino acid level rather than nucleotide level, with sequence homology between AII and these antagonists apparently resulting from one portion of AII being complementary to another. Such homology would account for the binding of these peptides to the AII receptor rather than AII itself. Also, the complementary peptide approach was recently used to purify the cellular receptor for fibronectin (40).

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