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ANGIOTENSIN II RECEPTORS IN CHROMATIN FRAGMENTS GENERATED BY MICROCOCCAL NUCLEASE

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SUMMARY: Rat liver nuclei were digested with micrococcal nuclease following incubation with ^{125}I-angiotensin II (AII) or with ^{125}I-AII and excess unlabeled hormone. Chromatin enriched in ^{125}I was solubilized after 3 min and was applied to a BIO-GEL A-5 M column. Labeled hormone was 40 - 60% displaceable by unlabeled hormone, in nucleoprotein eluting with a V/Vo near 1.9, indicating that these solubilized chromatin fragments contained specific receptors for AII. Furthermore, a discrete AII binding nucleoprotein particle was resolved on DNP gel electrophoresis. Additionally, binding to specific AII nuclear receptors appeared to bring about changes in chromatin structure consistent with the induction of transcriptional activity.

INTRODUCTION: Hormones coordinate metabolic activities by binding to specific receptor proteins located either on the cell membrane or within responsive cells. Since peptide hormones are believed to act primarily by binding to external membrane loci (1,2), very little effort has been directed toward the isolation and characterization of intracellular receptors, and in particular, nuclear receptors for peptide messengers. In the case of the peptide hormone angiotensin II (AII), however, evidence for specific nuclear AII receptors is increasing (3-5). Most recent is the demonstration of AII induced solubility alterations in chromatin which appeared to be due to conformational changes elicited by hormone binding i.e., the hormone enhanced the initial nuclease digestion kinetics (5). In the present papers we extend these studies with endonuclease digestion and present further evidence for specific AII receptors in chromatin.

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Binding of peptide hormones to external membrane sites is probably the first step in a sequence of steps that lead to a hormone's final action. What follows this initial binding is poorly understood but internalization and the generation of peptide fragments are likely consequences. Such internalized fragments could, in theory, interact directly with subcellular components, therefore, it is of interest to identify potential intracellular and nuclear receptors. In this report, evidence for specific AII receptors in chromatin fragments comes from displacement of binding by unlabeled AII and from DNP gel electrophoresis. From other laboratories there is evidence to suggest the intracellular generation of angiotensin II in many tissues (6-9) thereby raising the possibility that some of the locally synthesized peptide could bind to specific intracellular receptors.

MATERIALS AND METHODS:

Isolation of Nuclei, Nuclease Digestion and Fractionation of Chromatin. Procedures have been previously described (5, 10-11).

Hormone Incubation: Washed nuclear pellet obtained from rat liver was suspended in hormone incubation buffer (150 mM NaCl 10 mM Tris, 5 mM DTT, 1 mM PMSF, pH 7.5) to the desired concentration prior to the addition of either 10^{-9} M labeled AII (5-L isoleucine) [tyrosyl ^{125}I, specific activity 1880 uCi/ g, New England Nuclear] or 10^{-9}M labeled AII plus 10^{-6}M unlabeled AII. Hormone incubation was carried out at room temperature for 10 min. After incubation the hormone treated nuclear suspension was suspended to the desired concentration in digestion buffer (0.25 M sucrose, 15 mM Tris, 15 mM NaCl, 60 mM KCl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, pH 8.0 adjusted with solid cacodylic acid) by homogenizing at low speed (Willem's Polytron Homo genizer) for 15 sec. The nuclear suspension (>30 A_{260}/ml) was treated with micrococcal nuclease (Sigma Chemical Co., St. Louis, Missouri, 0.059 units of activity per unit of absorbance at 260 nm) as indicated in the results. In one experiment, nuclei were digested with micrococcal nuclease and the pellet removed prior to incubation of the solubilized supernatant with labeled or labeled and unlabeled hormone. (See results, Figure 3B.) In all experiments, digestion was terminated by transferring to an ice bath without the addition of EDTA.

DNP (deoxynucleoprotein) gel Electrophoresis: Following a 3 min digestion of labeled AII treated nuclei and gel filtration, samples from a Bio-Gel A-5M column (100-200 MESH, BIO-RAD Laboratories) with V/Vo 1.40-1.60 (mononucleosomes) and V/Vo 1.84-1.94 (leading edge linker DNA region) were pooled and were designated as sample B and $^{125}I\ B_2$ respectively. These two samples were dialyzed into dilute NH_4 formate, frozen and lyophilized, dissolved in a small volume of dilute NH_4 formate and re-lyophilized prior to resuspending in a small volume of 10 mM Tris-borate (electrophoresis buffer). Samples were then applied to a bi-directional DNP gel (1/16 in thick flatbed: 4% Acr (1/30 Bis), 0.5% Agarose) and electrophoresed adjacent to DNA markers. The gel was stained with fluorescent dye (ethidium bromide), photographed, drained, blotted, dry, adhered to used X-ray film, over-laid and sealed with plastic wrap, frozen and autoradiogrammed at -70°C with the use of an enhancing screen (exposure time 3 weeks). Nuclei treated

with labelled angiotensin II plus 10^{-6}M unlabeled hormone to verify displacement were similarly studied on DNP gels.

RESULTS: The profile (A_{254}) in Figure 1 (bottom) was obtained from nuclei incubated with labeled AII prior to digestion with micrococcal nuclease at 4°C for 4 hrs. Peaks A and B_1 were previously characterized by protein and DNA electrophoresis as oligonucleosomal and mononucleosomal nucleoprotein fragments, respectively (11). Protein electrophoresis of Peak B_2, which appeared when chelating agents were omitted, showed the presence of several H_1 species, several non-histone proteins but were deplete of core histones; DNA electrophoresis demonstrated that native B_2 DNA has a length of about 46 base pairs, suggesting that B_2 nucleoprotein probably corresponds to internucleosmal chromatin, that is, linker DNA (11). Peaks labeled C_1,C_2, and C_3 were soluble in alcohol, and contained non-dialyzable oligonucleotides; therefore, they are presumed to be products of extensively

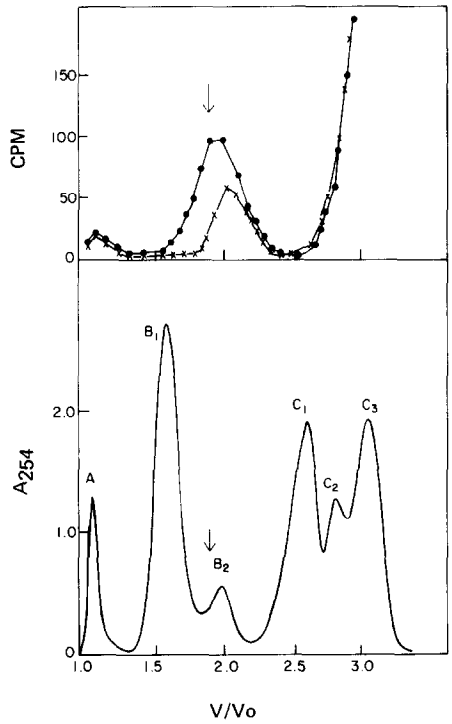

Figure 1 BIO-GEL A-5 M column elution profile of chromatin incubated with 10^{-9}M AII prior to digestion with micrococcal nuclease at 4°C for 4 hrs (bottom). Distribution of labeled AII (●) or labeled AII in presence of 1000 fold excess unlabeled AII (x) (top). ↓ denotes V/Vo 1.9

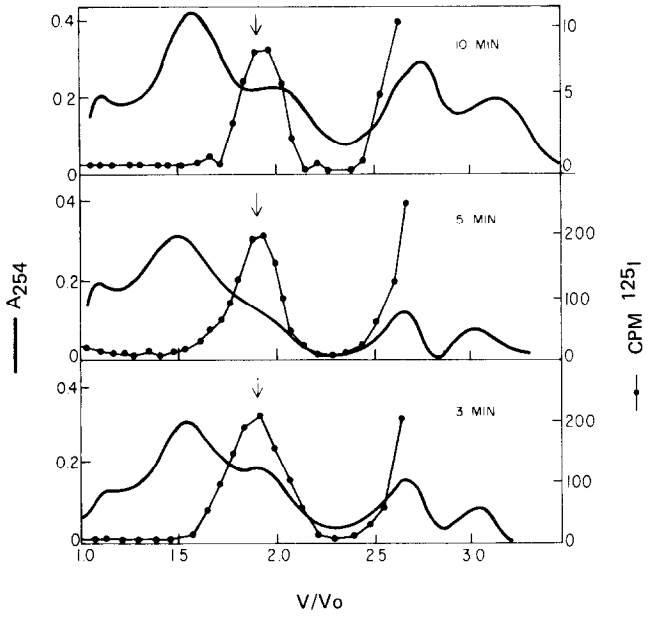

Figure 2 BIO-GEL A-5 M column elution profile of chromatin incubated with 10^{-9}M ^{125}I-AII prior to digestion with micrococcal nuclease at 30°C for indicated times. ↓ denotes V/Vo of 1.9.

digested chromatin (11). On shorter digestions, only one or two C peaks are resolved (See Figures 2 and 3).

Labeled AII appeared in the profile near the leading edge of the B_2 peak; the AII peak maximized at V/Vo 1.9 (Figure 1, top). In the presence of 10^{-6}M unlabeled AII, bound ^{125}I AII was displaced in this region by approximately 40%, suggesting that the hormone is binding to a specific receptor. When nuclei were digested for shorter periods (3-5 min) and at higher temperatures (30°C), labeled AII was again detected at the leading edge of peak B_2 (Figure 2). Nuclease digestion in the presence of a large excess of hormone influenced the specific activity of the ^{125}I-AII bound species but did not alter the position of the maximum (Figure 2, digestion of 3 and 5 min as compared to digestion of 10 min in the absence of excess labeled AII).

In another experiment, nuclei were incubated with labeled AII prior to a 3 min digestion at 30°C, and the ^{125}I-AII bound species at V/V 1.9 was displaced approximately 60% by an excess of unlabeled AII (Figure 3, A). Longer digestions (30 min) at 30°C showed less displacement (30-40%, data

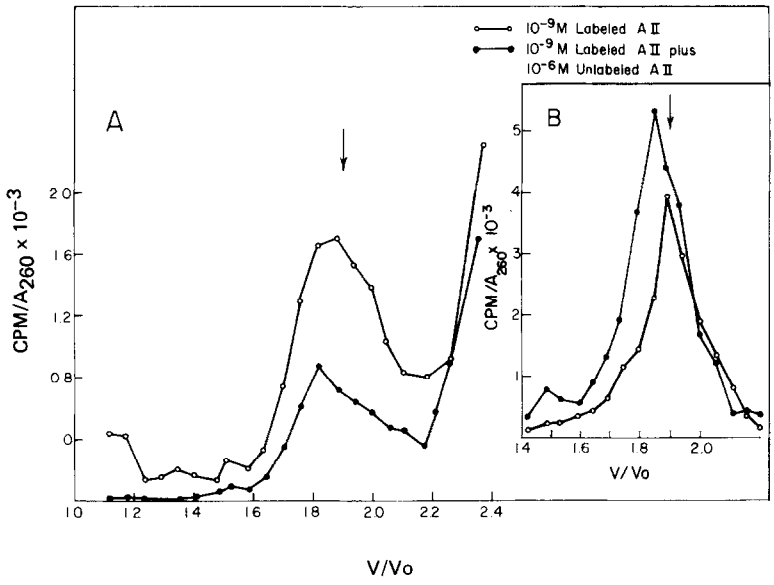

Figure 3 BIO-GEL A-5 M column elution profile of chromatin incubated with hormone prior to digestion (A) or to solubilized chromatin after digestion (B). ↓ denotes V/Vo of 1.9. The ordinates (CPM/A_{260}) refer to the ^{125} I activity in the column fraction normalized to the amount of chromatin (A_{260}) applied to the column.

not presented). Thus, fragments initially generated by micrococcal nuclease appear to contain a large number of specific receptors; upon longer digestions, these specific receptors are degraded and subsequent hormone binding appears to be non-specific. When incubation with hormone occurred after digestion, subsequent treatment with excess unlabeled AII showed no displacement of binding near V/Vo of 1.9, but there was, instead, enhanced binding in this portion of the profile (Figure 3). It should be emphasized that solubilized chromatin, in this experiment, was removed from the nuclear pellet prior to hormone treatment. Hence, chromatin fragments generated by the digestion of intact nuclei seem to contain regions where hormone receptor proteins are more concentrated and micrococcal nuclease preferentially attacks these regions.

Fractions from a portion of the 3 min profile in Figure 2 which appeared to contain specific receptor (V/Vo 1.81-1.94) were pooled and subjected to DNP gel electrophoresis. The results in Figure 4 show the resolution of a discrete particle (lane 2) which migrates slower than the

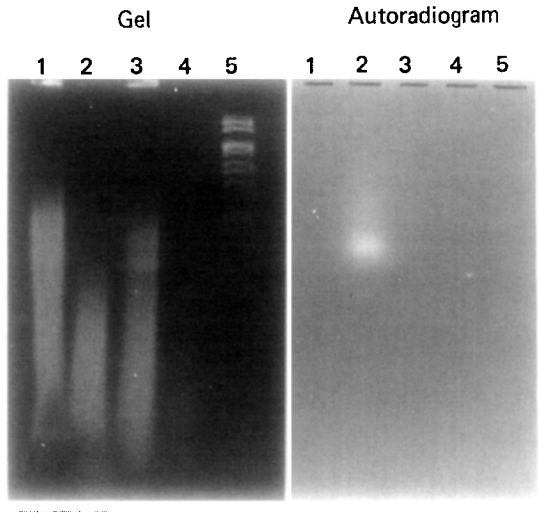

Figure 4 DNP GEL-Autoradiogram of fractions from 3 min digestion (Figure 2). Lanes (1) 10 µl B_1 (2) 10 µl ^{125}I-B_2 (3) 2 µl B_1 (4) 2 µl ^{125}I-B_2 (5) Marker DNA. [B_1 sample included V/Vo 1.4-1.6 and ^{125}I-B_2 included V/Vo 1.81-1.94; corrected counts/min for samples B_1 and ^{125}I-B_2 were 43 and 550 respectively.] Only that anodic half of the bidirectional gel is shown; neither stainable nor labeled material migrated in the other direction.

smear of fluorescence associated with the B_2 species, and appears to have the approximate mobility of mononucleosomes (lane 1). This nuclear particle is apparently more "compact" than other mononucleosomes in that it runs slower on A-5 M columns; however, the labeled particle could be completely unrelated to mononucleosomes. When nuclei were incubated with labelled AII plus 10^{-6}M unlabeled AII, DNP gels revealed virtually no tracer in the spot characteristic of this peptide, indicating that angiotensin II binding to this moiety is displaceable (data not presented). That the labeled particle may be subnucleosomal is suggested by its A-5 M chromatography (11); its mobility in DNP gels (slower than the B_2 fluorescent smear) suggests that it may have a lower charge than other species of the B_2 region previously associated with "linker" (11). The differential migration of the AII-labeled particle in different media provides the opportunity to purify and characterize both protein and DNA components of the particle; these experiments are in progress.

DISCUSSION: Hormone interactions with active regions of native chromatin enhance the accessibility of DNA to RNA polymerase, thereby increasing RNA

initiation sites (12-15). These hormone dependent effects are brought about, at least in part, by changes in chromatin structure. Evidence for such a structural change mediated by AII is the reported enhancement of nuclease susceptibility of chromatin upon AII addition (5). Other investigators have noted similar effects after treating tissue slices with the peptide hormone TSH (14,16). In the present report, evidence is presented for specific binding to AII nuclear receptors which could lead to such a conformational change.

Conformational change implicit from the present results follows from the observation that specific (displaceable) binding is found in chromatin fragments from AII-treated nuclei, and these fragments are preferentially (rapidly) generated by nuclease. In contrast, if only the solubilized portion of digested chromatin is hormone treated, there is a relative paucity of specific receptors. A rapid production of hormone-bound chromatin fragments by micrococcal nuclease could be expected, since it has been repeatedly demonstrated that this enzyme preferentially digests regions of chromatin enriched in transcribed genes (12-15). The inherently different responses of nuclei and digested chromatin to hormone treatment not only imply a structural change mediated by the hormone, but also might suggest a non-uniform distribution of AII receptors in chromatin. The data show that binding appears to be predominantly located in chromatin areas sensitive to micrococcal nuclease digestion, a condition likely associated with enhanced transcriptional activity and in regions of chromatin harboring hormone receptors. Thus, angiotensin II appears to increase the solubilization of its own receptors.

The specificity of angiotensin II binding in nuclei is also supported by the relatively discrete species found in DNP electrophoreses. We tentatively identify this species as a subnucleosomal particle, although further studies are needed to physically and biochemically characterize the species.

In addition, other current studies in this laboratory suggest that divalent metals mediate both specific binding and conformational changes in chromatin from AII-treated nuclei. It is of interest to understand and explain precisely how metals are involved in these processes, since this might provide critical insight into the mechanism(s) by which hormones function.

Finally, there are other recent reports of nuclear peptides capable of binding to DNA with possible regulatory effects in chromatin (17). It may well be that angiotensin exerts a similar nuclear regulatory function. In this regard it should be noted that both renin and angiotensin are found in multiple somatic cells, thereby raising the possibility that intracellular synthesized angiotensin II could play a role in cellular homeostasis.

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