Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 336-341

PHOSPHORYLATION AND NUCLEAR PROCESSING OF THE ANDROGEN RECEPTOR

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Received July 20, 1990

SUMMARY Although transformed androgen receptor (AR) complexes derived from cytosol and nuclear AR complexes have been shown to bind with high affinity to nuclei and DNA, we have shown that the binding characteristics of the two receptor populations to rat ventral prostate nuclei are different. To account for these differences, we investigated the possibility that the two receptor populations differed in phosphorylation status. Significantly, an anti-phosphotyrosine antibody immunoprecipitated androgen binding from the nuclear AR preparation but not from the transformed cytosolic receptor preparation. These studies suggest that (i) further processing of the AR complex takes place after it has become transformed, and (ii) phosphorylation of the complex is one modification which occurs during the processing of the nuclear receptor.

Transformation has been defined as the process by which the steroid receptor is converted to a nucleus-, DNA- or polyanion-binding form (1). Many experimental manipulations of the untransformed AR can result in its transformation, i.e. enhanced ability to bind to nuclei or DNA. However the molecular events underlying the transformation process are still being investigated. Moreover, we do not know whether the *in vitro* alteration of the AR during transformation is the only processing of the receptor required or whether the receptor is further processed within the nucleus to modulate its interactions with other components of the nucleus. In this paper we show that the AR complexes prepared from the nuclear compartment of the rat ventral prostate contain phosphotryrosine residues which are recognized by a polyclonal anti-phosphotyrosine antibody, whereas the transformed AR complex prepared from the cytosolic compartment is not recognized by this antibody. This is the first demonstration of the presence of phosphotyrosyl residues on nuclear but not on transformed cytosolic ARs and suggests that tyrosine phosphorylation may be required for nuclear interactions of the androgen-receptor complex.

MATERIALS AND METHODS

Preparation of Nuclei Nuclei were prepared exactly as previously described (2).

Preparation of Labelled AR Complexes AR complexes were incubated with 30 nM [^{3}H]-DHT for 18 h at 4°C in the presence and absence of 1000-fold excess unlabelled DHT. Unbound steroid was removed with charcoal: dextran.

0006-291X/90 $1.50

Preparation of Nuclear AR. The nuclear AR preparation was as described (3).

Preparation of Cytosolic Receptor. Partially purified cytosolic receptor (4) and purified cytosolic receptor (5) preparations were obtained as described.

Binding of AR Complexes to Purified Nuclei Nuclei were incubated with [^{3}H]-DHT-labelled AR complexes at 4°C for 2 h. Following the addition of 1 ml of 10 mM Tris-HCl, pH 7.4, the reaction was stopped by centrifuation for 2 min in a microfuge. The pellets were washed three more times. The tip of the tube was cut off and placed in 500 µl NCS tissue solubilizer and 5 ml Ready-Solv. The samples were incubated at 40°C for 2 h before determination of radioactivity.

Immunoprecipitation of AR Fractions using Rabbit Polyclonal Anti-Phosphtyrosine Antibody The description of the antibody and the immunoprecipitation protocol have been described previously (6).

RESULTS

Characterization of Binding of Transformed Cytosolic and Nuclear Receptors to Nuclei. The presence of NaCl was found to be required for specific binding of receptor complexes to nuclei. In 0.15 M NaCl, saturable specific binding of nuclear and transformed cytosolic AR complexes to nuclei prepared from 22 h castrated rats was observed (Fig. 1). There were calculated to be approximately 9000 specific binding sites for nuclear ARs per nucleus prepared from 22 h castrated rats and 600 sites for cytosolic receptors. Therefore, nuclei possess about 15-fold more sites for the nuclear AR than for the transformed cytosolic AR. Addition of cytosolic extracts with no receptor to assay tubes, in order to maintain constant putative inhibitor concentrations during an experiment, had no effect on binding of the receptor to nuclei. Thus, cytosolic inhibitors of binding of the receptor to the nucleus do not appear to be acting in this system.

Immunoprecipitation of Nuclear and Transformed Cytosolic ARs with an Anti-Phosphotyrosine Antibody Differences in their binding to nuclei suggested that nuclear ARs differ from transformed cytosolic receptors. As we had previously shown that the nuclear AR could be precipitated by an anti-phosphotyrosine antibody (3), we then investigated whether

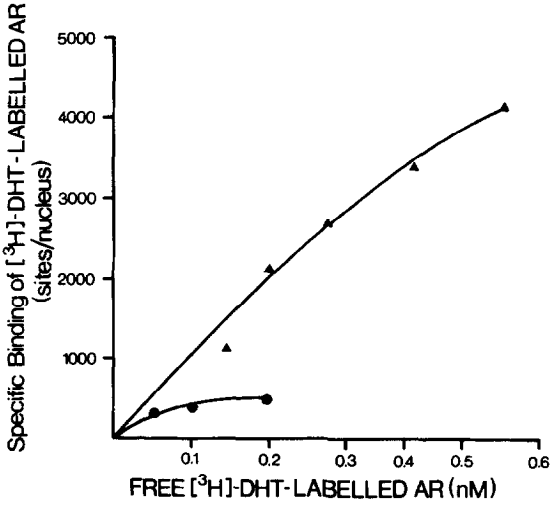


Fig. 1. Specific binding of AR complexes to ventral prostate nuclei prepared from 22 h castrated rats. Nuclei (cAR studies-11.4X10^6 nuclei; nAR studies-12.2X10^6 nuclei) prepared from 22 h castrated rats were incubated with partially purified nuclear (▲) or cytosolic AR complexes (●) labelled with [^{3}H]-DHT in buffer containing 0.15 M NaCl. The binding assay was done as described in Materials and Methods. Specific binding was calculated as the difference between total and non-specific binding.

Table 1. Immunoprecipitation of androgen receptor preparations with polyclonal anti-phosphotyposine antibody

Treatment	Specifically Bound [^{3}H]-DHT (fmol/mg protein) cAR	nAR
Non-immunoprecipitated androgen receptor	150	465
Anti-phosphotyrosine antibody	4±1	167±15
Pre-immune antiserum	-	50±7
IgG Sorb only (no antibody)	-	39±4

The androgen receptor fractions were labelled with [^{3}H]-DHT and either polyclonal anti-phosphotyrosine antibody, polyclonal pre-immune serum or IgG Sorb alone as described. Each value is the mean ± SEM from at least three determinations.

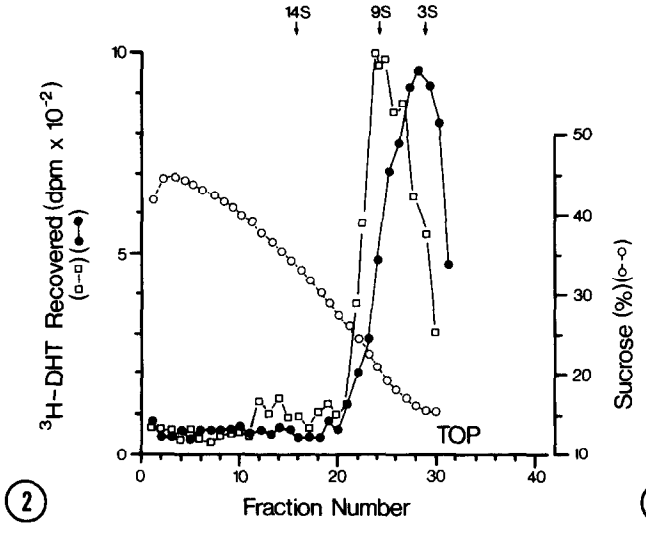
purified transformed cytosolic AR could be immunoprecipitated by the antibody. Table 1 shows that whereas 36% of the nuclear AR could be immunoprecipitated, only 3% of the transformed cytosolic receptor could be immunoprecipitated. We verified that no phosphatase activity was detected in the cytosolic receptor fraction when incubated for 30 min at room temperature in the presence of 20 mM Tris-HCl, pH 7.4, containing 1.5 mM EDTA, 10% glycerol (v/v), 25 mM monothioglycerol, 20 mM molybdate and 8 mg/ml p-nitrophenyl phosphate. The simplest explanation of the immunoprecipitation data is that the antibody cross-reacts with the nuclear AR or a closely associated protein. To gain more evidence for this we characterized by sucrose density gradient centrifugation the interactions of the antibody with partially purified nuclear AR. Figure 2 shows that the partially purified nuclear AR has a sedimentation coefficient of about 3S. After incubation with the rabbit polyclonal anti-phosphotyrosine antibody, the receptor complexes sedimented primarily at about 8-9S with a much smaller peak at about 14-19S. We were unable to detect a shift in the purified transformed cytosolic receptor after immunoprecipitation with anti-phosphotyrosine antibody (results not shown).

Figure 3 shows the electrophoretic profile of phosphotyrosyl proteins following immunoprecipitation of the nuclear AR. Few proteins were recovered; most notable were the peptides with relative mobilities 35, 17, 16.5, 15.5 (as a doublet) and 14.3 kDa. We have previously described a 35 kDa nuclear protein which may be a proteolytic fragment of the AR.

Analysis of the Nuclear AR by Isoelectric Focussing. Since only a third of the nuclear AR was immunoprecipitatable, we investigated by isoelectric focussing the possibility of different isoforms of the nuclear AR. The isoelectric point of the native form of the nuclear AR was determined by flat-bed polyacrylamide gel isoelectric focussing at 4°C. Figure 4A shows that the nuclear AR was a basic protein and that there were at least three forms or isotypes of the nuclear AR, possessing pIs of 8.0, 7.5 and 7.2 respectively. Duplicate samples of the partially purified AR were incubated with [^{3}H]-DHT in the presence of 1000-fold excess unlabelled DHT and focussed simultaneously; in each case the radioactivity associated with each of the three putative isoforms of the receptor was displaceable with non-radioactive DHT. Figure 4B shows that the three peaks of radioactivity could not be detected from partially purified nuclear receptor fractions prepared from 20-24 h castrated rats.

DISCUSSION

For some time now, controversy has existed about whether steroid hormones first bind to receptor in the cytoplasm and then undergo transformation to a DNA binding form before



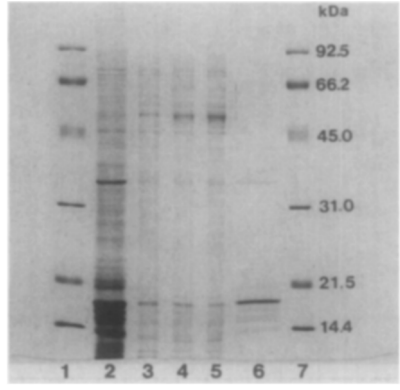


Fig. 2. Sedimentation analysis of the interaction of nuclear AR with rabbit polyclonal anti-phosphotyrosine antibody under high ionic conditions. Partially purified nuclear AR labelled with [^{3}H]-DHT (75 fmol) was incubated with (□) and without (●) 25 µl of rabbit polyclonal anti-phosphotyrosine antibody in 10 mM TES-NaOH, pH 7.5, containing 1.5 mM EDTA-4Na, 0.5 mM mercaptoethanol and 0.4 M KCl for 4.5 h at 4°C. The samples (250 µl) were layered onto linear 10-50% sucrose density gradients in the same buffer and centrifuged in a Beckman SW55 rotor for 16 h at 35,000 rpm at 4°C. Fractions of 150 µl were collected from the bottom of the tubes and assayed for radioactivity.

Fig. 3. Electrophoretic analysis of nuclear AR immunoprecipitated with polyclonal anti-phosphotyrosine antibody. Nuclear AR (100 µg protein) was incubated with 10 µl antibody for 1.5 h at 4°C. Protein A was added for 30 min before washing and addition of p-nitrophenyl phosphate. The supernatant (lane 6) and pellet (lane 5) obtained after centrifugation were analyzed by SDS-PAGE (12% gel). Lanes 1 and 7 -BioRad low molecular weight markers. Lane 2 -untreated nuclear AR. Controls are also shown in which the receptor fraction was incubated with only Protein A (lane 3) or antibody (lane 4).

translocation into the nucleus to effect their action (7) or whether steroids interact with the receptor for the first time within the nucleus where transformation takes place (8,9). Regardless of the *in vivo* localization of the receptor, receptor obtained from the cytosol after subcellular fractionation corresponds to a non-DNA binding, non-transformed receptor which has an *in vivo* counterpart and nuclear receptor corresponds to the DNA binding form. Thus, the term cytosolic receptor used in this report is an operational term which designates a population of receptor which may or may not truly reside in the nucleus, but which is recovered in the cytosol after subcellular fractionation. Our aim was to determine whether transformation of the receptor recovered from the cytosol yielded receptor identical to nuclear receptor.

Others (10, 11) have reported that non-specific binding, which might mask the presence of nuclear acceptor sites, is decreased when 0.15-0.20 M KCl is present in the binding assay. We confirmed this finding. Potential binding sites associated with intact nuclei include chromatin (12), DNA (13), basic nuclear proteins (14), ribonucleoprotein particles (15) and nuclear envelopes (16). Significantly, we found that there were 15-fold fewer sites for the transformed cytosolic AR than for nuclear receptors on rat ventral prostate nuclei. No previous studies compare the binding of both transformed cytosolic and nuclear forms of the AR to nuclear fractions. Furthermore, the finding is of significance as it suggests that

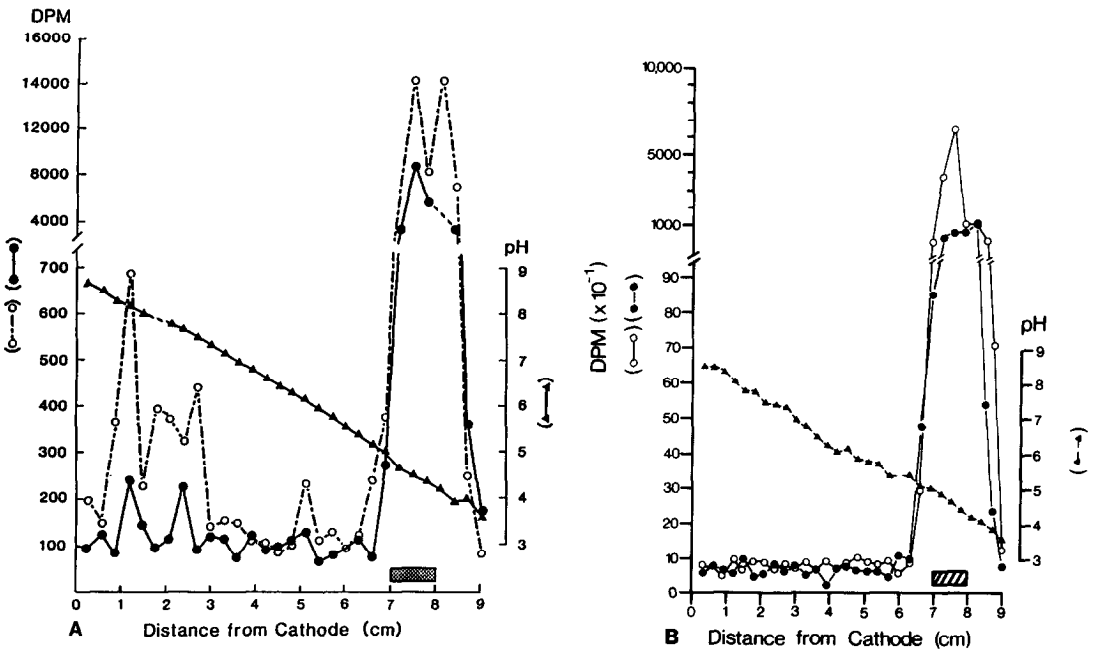


Fig. 4. Analysis of partially purified nuclear AR by isoelectric focussing. Partially purified nuclear AR fractions from (A) intact rats or (B) 24 h castrated rats were incubated with 30 nM [^{3}H]-DHT in the presence (●) and absence (○) of 1000-fold excess unlabelled DHT for 18 h at 4°C. Fifty µg of protein was applied with filter paper onto the surface of preformed Ampholine PAGplate (pH 3.5 - 9.5) polyacrylamide gels and isoelectric focussing was initiated. After 30 min the filter paper was removed and the isoelectric focussing was continued for an additional 60 min. The gel was then cut into 3 mm slices, placed in scintillation vials and incubated with 200 µl of 50% H_2O_2 at 70°C overnight. The recovery of radioactivity was measured after the addition of 10^3 U of catalase and 10 ml of Ready-Solv.

processing of the receptor takes place in addition to the alterations involved in transformation of the receptor which allow the receptor to bind to DNA.

In this paper we show that an anti-phosphotyrosine antibody causes a shift in the sedimentation profile of the nuclear AR, lending support to the notion that the antibody is cross-reacting with the receptor itself or a closely associated protein (17). We have also compared the immunoprecipitation of the nuclear AR fraction to that of a purified cytosolic receptor fraction. Significantly, the antibody was unable to immunoprecipitate [^{3}H]-DHT binding activity from the cytosolic fraction nor did it cause a shift in the cytosolic receptor from its typical 8S position on a sucrose gradient (results not shown).

Our recovery of multiple forms of the nuclear AR by isoelectric focussing confirms other investigations of nuclear AR (18, 19). Cytosolic AR prepared from the rat ventral prostate is an acidic protein possessing a pI of 5.8 (20). If the *in vivo* AR is a more basic entity, it would have a stronger interaction with the chromatin. In combination with our study, these results support the view that (a) the nuclear AR is a basic protein and (b) that it exists as multiple isoforms. These multipe isoforms may be involved in the regulation of physiologically significant phenomena.

Others have reported phosphorylation of steroid receptors. Many roles for the phosphorylation have been proposed, but as yet a function has not been proven. Moreover, a single type of phosphorylation reaction cannot account for the phosphorylation of steroid receptors as these proteins have been shown to be phosphorylated on serine (21), threonine (22)

and tyrosine (21) residues. We have shown that a population of nuclear glucocorticoid receptor is situated at the periphery of the nucleus and have proposed that this is receptor in transit across the nuclear envelope (23). Nuclear entry of proteins has been shown to require ATP. Perhaps receptor or a closely associated protein is phosphorylated during nuclear translocation.

In conclusion, we have shown that ARs derived from the cytosolic and nuclear compartments of the cell differ in their ability to bind to nuclei. We have shown with anti-phosphotyrosine antibodies that a significant proportion of the nuclear receptor or a protein closely associated with it appears to be a phosphotyrosine-containing protein whereas the transformed cytosolic AR complex does not. These data and our identification of three isoforms of the nuclear AR are consistent with a role for phosphorylation in further processing of the receptor after its transformation.

ACKNOWLEDGMENT

This investigation was funded by the National Research Council of Canada.

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