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DNA-BINDING AND NON-DNA-BINDING FORMS OF THE TRANSFORMED GLUCOCORTICOID RECEPTOR

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Summary—In this work we have probed the mechanism responsible for two non-DNA-binding states of the mouse glucocorticoid receptor. In the first case, transformed receptors were treated with hydrogen peroxide. It is known that oxidizing agents promote the formation of disulfide bonds in the glucocorticoid receptor, but it has not been determined what domains are involved in any disulfide bond formation that leads to inactivation of DNA-binding activity. We show here that hydrogen peroxide inhibits DNA-binding by the 15-kDa tryptic fragment containing the DNA-binding fingers with the same concentration dependency as it inhibits DNA-binding by the uncleaved receptor. This suggests that all of the effect of peroxide is on sulfhydryl groups within the zinc fingers. After dissociation (transformation) of cytosolic heteromeric glucocorticoid receptor complexes, only a portion (40–60%) of the dissociated receptors can bind to DNA-cellulose. We show that the 15-kDa tryptic fragment derived from the portion of transformed receptors that do not bind to DNA is itself competent at DNA-binding.

INTRODUCTION

We previously reported that hydrogen peroxide inhibits both transformation of glucocorticoid receptors to the DNA-binding state and DNA-binding by previously transformed receptors [1]. Silva and Cidlowski [2] have provided direct evidence that oxidizing agents promote the formation of intramolecular disulfide bonds in the glucocorticoid receptor, and inhibition of DNA-binding could result from such disulfide bond formation. A major question that has not yet been answered is whether disulfide bond formation occurs between the DNA-binding domain and another domain of the receptor or whether formation of disulfide bonds within the DNA-binding domain accounts for inhibition of DNA-binding activity. In this work, we show that hydrogen peroxide inhibits DNA-binding by the 15-kDa tryptic fragment of the receptor, suggesting that disulfide bond formation within the DNA-binding domain accounts for the effect.

After transformation (activation) of the glucocorticoid receptor, two forms of the monomeric receptor can be distinguished by isoelectric focusing (pI $\approx$ 5.7 and 6.0–6.5) [3]. The charge heterogeneity has been localized to a M_r 26,500 fragment corresponding to residues 499–743 in the steroid-binding region of the human receptor, suggesting that some modification of the steroid-binding domain may regulate the ability of the receptor to bind to DNA [4]. Consistent with this proposal, we show here that the 15-kDa tryptic fragment [residues 374–505 (mouse)] from the non-DNA-binding fraction of the transformed mouse glucocorticoid receptor has DNA-binding activity.

EXPERIMENTAL

Chemicals

[6,7-^{3}H]Triamcinolone acetonide (42.8 Ci/mmol), and ^{125}I conjugated goat anti-mouse and goat anti-rabbit IgG were obtained from New England Nuclear (Boston, MA). Radioinert dexamethasone, goat anti-mouse and anti-rabbit IgG-horseradish peroxidase conjugate, were from Sigma Chemical Company (St Louis, MO). The BuGR2 monoclonal antibody against the glucocorticoid receptor was kindly provided by Dr Robert W. Harrison.

Cell culture and fractionation

L929 mouse fibroblasts were grown in monolayer culture in Dulbecco's modified Eagle medium supplemented with 10% bovine or calf

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serum. Cells were harvested in late log phase growth by scraping into Earle's balanced saline followed by a second wash and centrifugation at 500 *g*. The washed cells were resuspended in 1.5 vol of HEPES buffer (10 mM HEPES, 1 mM EDTA, pH 7.35 at 4°C) and ruptured by Dounce homogenization. Homogenates were centrifuged for 1 h at 100,000 *g*. The supernatant from this centrifugation was removed and is referred to as "cytosol".

DNA-binding assay

Cytosol was bound overnight at 0°C with 200 nM dexamethasone. To transform receptors to the DNA-binding state, cytosol containing steroid-bound receptors was incubated for 1 h at 25°C. Incubations containing hydrogen peroxide were prepared as indicated in the figure legends. For assay of DNA-binding, 100 μl aliquots of incubation mix were incubated for 1 h at 4°C with a 200 μl suspension of 12.5% DNA-cellulose. The pellets were washed 3 times with 1 ml of buffer and solubilized with SDS sample buffer and analyzed by SDS–PAGE as described below.

Receptor partial proteolysis

Cleavage of receptor was performed in whole cytosol after transformation and before other treatments essentially as described previously [4]. Freshly prepared TPCK-treated trypsin (200 μg/ml final concentration) was added to cytosol and allowed to incubate on ice for 1 h. To stop proteolysis, 1 mg/ml soybean trypsin inhibitor was added for 30 min on ice.

Immunoadsorption of receptor

The BuGR anti-receptor antibody (hybridoma culture supernatant) or non-immune mouse IgG (1 mg/ml) was preadsorbed to protein A-Sepharose by rotation with the Sepharose beads for 2 h at 4°C and the beads were then washed 3 times with 1 ml aliquots of TEG buffer [10 mM TES, 4 mM EDTA, 10% (w/v) glycerol, 50 mM NaCl, pH 7.6]. Aliquots (100–400 μl) of cytosol were mixed with preadsorbed antibody pellets, rotated for 1 h at 4°C, and the pellets were washed 3 times with 1 ml aliquots of TEG buffer.

Gel electrophoresis and immunoblotting

SDS–PAGE was performed in 7 or 10% slab gels essentially as described previously [4]. Gels were cooled to 4°C during electrophoresis. All samples were extracted from DNA-cellulose or protein A-Sepharose by boiling in SDS sample buffer with or without 10% β-mercaptoethanol. M_r standards were: myosin, M_r 205,000; β-galactosidase, M_r 116,000; phosphorylase B, M_r 97,000; bovine serum albumen, M_r 66,000; egg albumen, M_r 45,000; glyceraldehyde-3-phosphate dehydrogenase, M_r 36,000; carbonic anhydrase, M_r 29,000; trypsinogen, M_r 24,000; soybean trypsin inhibitor; M_r 21,000, and α-lactalbumin, M_r 14,500.

Immunoblotting was carried out by transferring proteins from acrylamide slab gels to Immobilon-P membranes under transfer conditions of 2 h × 0.6 A for the intact receptor and 16 h × 0.1 A for the 15-kDa tryptic receptor fragment. Immobilon-P membranes were then incubated with 1% BuGR antibody against the glucocorticoid receptor followed by a second incubation with ^{125}I-labeled goat anti-mouse IgG.

RESULTS AND DISCUSSION

To determine if hydrogen peroxide inhibits DNA-binding through a direct action on the DNA-binding domain, both intact and trypsin-cleaved receptors in L cell cytosol were treated

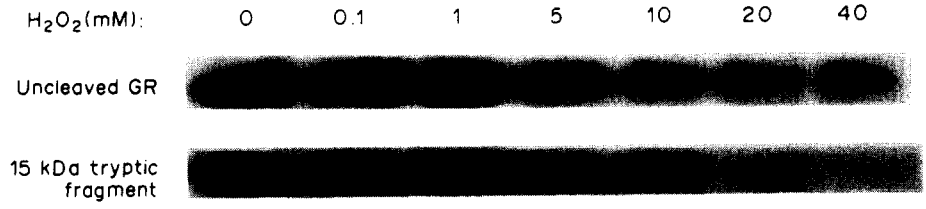

Fig. 1. Hydrogen peroxide is equally effective at inhibiting the DNA-cellulose binding activity of the uncleaved glucocorticoid receptor and the 15-kDa tryptic fragment of the receptor. Aliquots of cytosol (230 μl) were transformed to the DNA-binding state and incubated (or not) with trypsin to cleave the glucocorticoid receptor. Samples were then incubated with increasing concentrations of hydrogen peroxide for 1 h at 0°C and bound to DNA-cellulose. After washing, the DNA-bound proteins in the cellulose pellets were solubilized in sample buffer, resolved on SDS–PAGE, transferred to Immobilon-P membranes, and blotted with BuGR anti-receptor antibody, followed by ^{125}I-labeled counterantibody and autoradiography. The concentration (mM) of peroxide is indicated above each lane.

with various concentrations of the oxidizing agent for 1 h at 0°C. Receptors were then bound to DNA-cellulose and the amount of receptor or receptor fragment binding to DNA is shown in Fig. 1. It is clear that under these conditions where the receptor is in cytosol hydrogen peroxide inhibits DNA-binding by the 15-kDa tryptic fragment with essentially the same concentration dependency as it inhibits DNA-binding by the intact receptor. In other experiments, we have shown that peroxide treatment does not lead to cleavage of receptor protein in cytosol [6]. The inhibition of DNA-binding activity caused by 20 mM hydrogen peroxide treatment of the 15-kDa fragment can be overcome by incubating samples for many hours at 0°C with 30 mM dithiothreitol and 1 mM $ZnCl_2$ [7].

The 15-kDa tryptic fragment [8] of the glucocorticoid receptor contains the two zinc "fingers" and the BuGR epitope [6]. This fragment contains 11 cysteines, of which 8 are involved in the tetrahedral coordination of zinc. One mechanism by which the oxidizing agent could inhibit DNA-binding is by promoting the formation of disulfide bridges between vicinal thiols, resulting in the elimination of zinc and disrupting the finger structure, which is required for receptor binding to DNA [9]. The fact that hydrogen peroxide inhibits DNA-binding by the 15-kDa fragment strongly suggests that disulfide bond formation between the DNA-binding domain and another domain, such as the hormone-binding domain, does not account for redox inhibition of DNA-binding by the intact receptor. As an alternative to disulfide bond formation, it is possible that reversible oxidation products, such as sulfenic or sulfinic acids are formed, as has been proposed for redox regulation of Fos and Jun-binding to DNA [10].

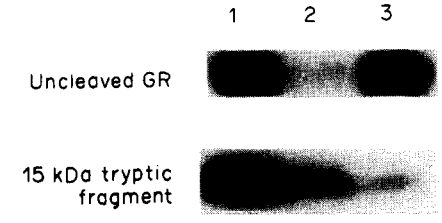

Fig. 2. The 15-kDa tryptic fragment derived from the non-DNA-binding fraction of the transformed glucocorticoid receptor has DNA-binding activity. Cytosol containing dexamethasone-bound glucocorticoid receptors was incubated 1 h at 25°C to transform the receptors to the DNA-binding state. The cytosol was then extracted twice for 1 h at 4°C with DNA-cellulose to remove the DNA-binding fraction of receptor. Half of the DNA-cellulose-extracted cytosol was treated with trypsin to generate the 15-kDa tryptic fragment of the receptor. Uncleaved and cleaved receptors were then bound again to DNA-cellulose. After centrifuging out the DNA-cellulose-bound receptors, the unbound receptor was extracted from the supernatant by adsorption to protein A-Sepharose using BuGR anti-receptor antibody. Receptor or fragment in the DNA-cellulose and immune pellets was then solubilized in sample buffer and resolved by SDS–PAGE and immunoblotting. Lane 1, the receptor in the supernatant after the second DNA-cellulose extraction. The sample of lane 1 was then incubated a third time with DNA-cellulose and separated into DNA-bound (lane 2) and non-DNA-bound (lane 3) fractions.

In the experiment shown in Fig. 2, dexamethasone-bound receptors in L cell cytosol were transformed by heating and then bound to DNA-cellulose. About half of the receptors bound to DNA-cellulose, and the non-DNA-binding receptors in the supernatant were extracted a second time with DNA. Receptors that did not bind after two extractions with DNA-cellulose are shown in lane 1. The non-DNA-binding receptors were then cleaved with trypsin and incubated again with DNA-cellulose. As shown in lane 2 in the bottom half of Fig. 2, the 15-kDa fragment derived from the non-DNA-binding intact receptor (compare with lanes 2 and 3, top) binds to DNA. Thus, it would seem that the inability of this fraction of the transformed receptor to bind to DNA is not intrinsic to the DNA-binding fingers themselves. This leads us to propose that another domain (perhaps the hormone-binding domain as suggested by the data of Harmon *et al.* [4]) can block the DNA-binding domain in a portion of receptors after dissociation of the hsp90-containing receptor heterocomplex.

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