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# C-TERMINAL PROTEOLYSIS OF THE AVIAN 1,25-DIHYDROXYVITAMIN $D_3$ RECEPTOR

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Exposure of the 60 kDa chick intestinal 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) receptor to carboxypeptidase A resulted in a time dependent decrease in receptor hormone-binding; after 2 h, there was no detectable macromolecular-bound $1,25(OH)_2[^3H]D_3$. Upon DNA-cellulose chromatography of this preparation, a 56 kDa protein adsorbed to the column and eluted as a function of para-chloromercuribenzene sulfonate (a sulfhydryl blocking reagent). The 56 kDa fragment was detected by anti-receptor monoclonal antibodies via immunoblot technology. The $1,25(OH)_2[^3H]D_3$ eluted in the fall through fractions of the column. Thus, cleavage of up to 40 amino acids from the carboxy-terminus of the $1,25(OH)_2D_3$ receptor results in a protein which no longer binds to hormone, but retains its capacity to interact with DNA-cellulose and monoclonal antibody. These results represent novel biochemical evidence that allows us to orient the $1,25(OH)_2D_3$ binding domain near the C-terminus of the receptor. 

The receptor for 1,25-dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$) is a 60 kilodalton (kDa) protein which is thought to mediate its action by transcriptional activation of target genes (1,2). Limited enzymatic digestion of the $1,25(OH)_2D_3$ receptor has been used by us (3,4) and others (5) to gain insight into the structural/functional relationships of the receptor. Monoclonal antibodies produced against the receptor (6-8) have provided the means for us to determine that the DNA-binding and hormone-binding domains of the receptor can be separated by trypsin cleavage in an intact and functional state (3,4). Cleavage with an endogenous protease facilitated our mapping of the monoclonal antibody determinant between these two domains, close to or slightly within the DNA-binding region (4). Here, we report the amino (N), carboxy (C)-terminus orientation of the domains as elucidated by assaying the effect of carboxypeptidase

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cleavage of amino acids from the C-terminus of the receptor on the ability of the receptor to bind to hormone, DNA-cellulose, and monoclonal antibody.

## MATERIALS AND METHODS

Receptor Preparation. Chick intestinal extracts were prepared by homogenizing mucosa in 9 volumes (w/v) of KT-0.3 (0.3 M KCl, 0.01 M Tris-HCl, pH 7.4) with a glass-Teflon homogenizer at 0-4°C followed by centrifugation at 12,000 x g for 10 min. The buffer-soluble fraction (termed cytosol) was obtained by ultracentrifugation of the resultant supernatant at 200,000 x g for 45 min and then was lyophilized and stored at -80°C prior to use. Cytosol contained approximately 0.5 pmol of $1,25(OH)_2D_3$ receptor/mg of protein.

Assay for Receptor Hormone-Binding. Receptor-hormone complexes were quantitated by dextran-coated charcoal which was prepared by the method of Dokoh, et al (9). Aliquots of receptor solutions were first incubated with 4 nM $1,25(OH)_2[^3H]D_3$ and in parallel with both $1,25(OH)_2[^3H]D_3$ and a 100-fold excess of nonradioactive hormone for 2 h at 0-4°C.

Carboxypeptidase Digestion. Carboxypeptidase A-DFP was purchased as an aqueous suspension with toluene added. On the day of the experiment toluene was removed by spinning the suspension in a microfuge; the enzyme pelleted to the bottom of the tube and the toluene/water was drawn off. The enzyme was washed in distilled water and respun and then resuspended in distilled water to a known concentration and kept at 4°C. Hormone-labeled cytosol was raised in salt concentration to 0.5-0.8 M by the addition of KCl, and then aliquots were treated with carboxypeptidase (100-2000 units/ml) for various time intervals in a shaking water bath maintained at 25°C.

DNA-Cellulose Chromatography. DNA-cellulose was prepared by a modification of the technique described by Alberts and Herrick (10). Columns were packed to 2.5 x 3 cm and equilibrated in KT-0.05 prior to use and chromatography was carried out as outlined previously (11). After washing in low salt buffer at 0.2 ml/min samples were eluted at 1 ml/min via 1 mM para-chloromercuribenzene sulfonate (pCMBS) and collected in 3 ml fractions.

Polyacrylamide Gel Electrophoresis & Immunoblotting. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli (12). Protein samples were precipitated with a 6.5% final concentration trichloroacetic acid (TCA) to concentrate the protein. Samples were run on SDS-polyacrylamide gels and then electrophoretically transferred from the gel to a nitrocellulose membrane (13). Transfer was carried out at 4°C and the nitrocellulose membranes were subjected to immunoblot analysis using anti-receptor monoclonal antibodies (9A7γ), anti-9A7γ anti-serum, and radioiodinated protein A (30-40 μCi/μg, 75,000 cpm/ml) and then autoradiographed as detailed previously (4).

## RESULTS

Chick intestinal cytosol labeled with $1,25(OH)_2[^3H]D_3$ was exposed to carboxypeptidase A at 25°C and aliquots were taken at the initial time point and at one hour intervals, quenched with carboxypeptidase inhibitor, and assayed for specific hormone-binding by the dextran-coated charcoal method. Fig. 1 shows that the specific binding of $1,25(OH)_2[^3H]D_3$ to the receptor decreases with

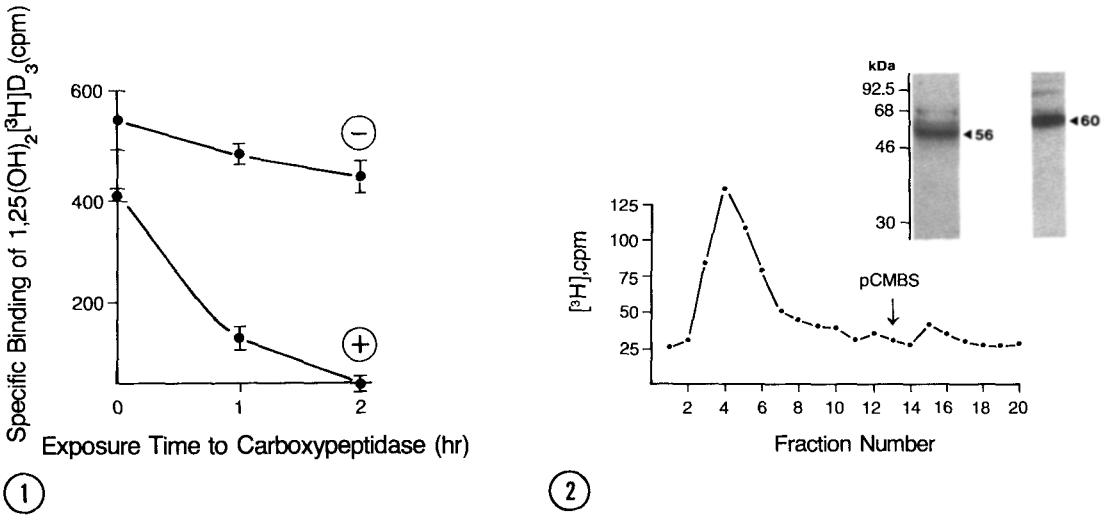


Fig. 1. Effect of Carboxypeptidase Treatment on Hormone-Binding of the $1,25(OH)_2D_3$ Receptor. Cytosol was labeled with 4 nM $1,25(OH)[^3H]_2D_3$ (90 Ci/mmol) with or without the presence of a 100-fold excess of $1,25(OH)_2D_3$ at 4°C for 2 h. Both preparations were raised to 0.8 M KCl, divided, and either were untreated (-) or exposed to 500 units carboxypeptidase/ml (+) at 25°C. Reactions were terminated by the addition of carboxypeptidase inhibitor at the indicated time points.

Fig. 2. DNA-Cellulose Chromatography and Immunodetection of Carboxypeptidase-Treated $1,25(OH)_2D_3$ Receptor. Hormone-labeled cytosol was treated with carboxypeptidase (500 units/ml) for 2 hr as described in Fig. 1. The preparation was equilibrated in KT-0 via application to a Sephedex G-25 desalting column and then chromatographed on DNA-cellulose. The column was washed in KT-0 (fractions 1-12) and then eluted in 1 mM pCMBS in KT-0 (fractions 13-20). Aliquots (0.10 ml) of each successive 3 ml fraction eluting from the column were assayed for tritium. Separate aliquots (2.5 ml) of each of the fractions also were subjected to immunoblot analysis and the immunoreactive fragment eluted by pCMBS is shown. The lane to the far right represents untreated cytosol prior to DNA-cellulose chromatography (14).

increased exposure time to carboxypeptidase; after 2 h there is no detectable hormone-binding. While the untreated receptor preparation also shows less hormone-binding after 2 h at 25°C, it does not exhibit the dramatic decrease characterized by the cytosol exposed to carboxypeptidase. Thus, this experiment demonstrates the apparent time dependent destruction of the $1,25(OH)_2D_3$ receptor's hormone-binding ability by carboxypeptidase.

Hormone-labeled cytosol was treated with carboxypeptidase for 2 h as in Fig. 1, applied to a DNA-cellulose column and then eluted with 1 mM pCMBS. The radioactivity profile in Fig. 2 shows that most of the $1,25(OH)_2[^3H]D_3$ eluting from the column is present in the fall through (unbound) fractions (fractions 1-12), while a small amount eluted in fractions 15-17 as a function of pCMBS. Individual fractions throughout the column were also assayed by immunoblot

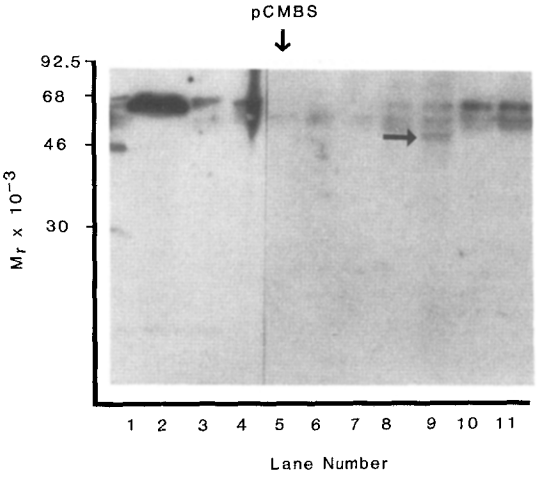


Fig. 3. DNA-Cellulose Chromatography and Immunodetection of Carboxypeptidase-Treated $1,25(OH)_2D_3$ Receptor. Hormone-labeled cytosol was treated with carboxypeptidase as in Fig. 2, except that the incubation time was 1 h. The fractions eluting from the DNA-cellulose column were immunoblotted. Lane 1, molecular weight standard proteins: phosphorylase β (92,500), bovine serum albumin (68,000), ovalbumin (46,000), and carbonic anhydrase (30,000); lane 2-4, fall through fractions; lanes 5-11, pCMBS elution. The signal at 66-68 kDa in lanes 2-4 and 9-11 is due to crossreactivity of an abundant protein with anti-9A7γ antiserum, i.e., it is independent of the primary monoclonal antibody (14).

technology. A unique signal at approximately 56 kDa is present in fraction 15, comigrating with the pCMBS eluant as indicated in Fig. 2. This species therefore binds to DNA and can be dissociated from DNA with pCMBS, just as the intact receptor can (15). The small amount of radioactivity that elutes in fraction 15 is most likely due to trace amounts of undigested native receptor of 58-60 kDa in the preparation that still retain hormone. Fig. 3 is an identical experiment to Fig. 2 except that cytosol has been treated with carboxypeptidase for only one hour. The immunoblot shows the elution by pCMBS of the undigested 60/58 kDa receptor doublet (arrowheads at right) as well as fragments of 56-57 kDa appearing in lane 9 (horizontal arrow). These results indicate that carboxypeptidase has cleaved up to 4 kDa from the C-terminus of the intact 60 kDa $1,25(OH)_2D_3$ receptor or up to 2 kDa from the 58 kDa species, which results in the generation of receptor fragments that have lost the ability to bind hormone, but not the capacity to interact with DNA or monoclonal antibody.

## DISCUSSION

Carboxypeptidase A, which preferentially attacks carboxy-terminus peptide bonds of amino acids with aromatic and aliphatic side chains, has been shown to

affect the $1,25(OH)_2D_3$ receptor's hormone-binding activity (Fig. 1), but not its ability to bind to DNA or monoclonal antibody (Figs. 2 and 3). Because carboxypeptidase A has little activity toward lysine, arginine, or proline residues, it is surprising that approximately 40 amino acids (~4 kDa) were cleaved from the 60 kDa receptor. We have previous evidence that the 58 kDa species is an endogeneous proteolytic product of the 60 kDa receptor that is particularily evident after DNA-cellulose chromatography (14). It is therefore reasonable that the carboxypeptidase-generated 56 kDa fragment arises from C-terminal cleavage of up to 2 kDa from the 58 kDa receptor form. Additionally, it does not seem plausible that the 2-4 kDa C-terminal region encompasses the entire hormone-binding domain. Rather, more probable interpretations of the data would be that the cleaved 2-4 kDa portion is either i) necessary for the tertiary stabilization of a less C-terminal hormone-binding domain or ii) an actual component of a larger hormone-binding "pocket" which is disrupted upon removal of the C-terminal amino acids. Regardless, from the present data we can conclude that the $1,25(OH)_2D_3$-binding domain is located C-terminal to the DNA-binding domain and the antigenic determinant.

There is ample precedent for this arrangement in the $1,25(OH)_2D_3$ receptor, as evidenced by data from other steroid and thyroid hormone receptors. The recent cloning (16) and transfection (17) of the glucocorticoid receptor indicate that the hormone-binding domain encompasses some 230 amino acids at the C-terminus of the molecule. A variant glucocorticoid receptor form has been identified through its cDNA that lacks the C-terminal 35 amino acids of the normal receptor and, because this species does not bind hormone, Weinberger *et al.* have concluded that the extreme C-terminus of the molecule is critical to hormone binding (18). Moreover, cloning of the c-*erbA* gene, the cellular counterpart of the viral oncogene, v-*erbA*, has also recently been accomplished (19,20). The 46 kDa c-*erbA* protein has been shown to bind thyroid hormone (19,20) and has 89% amino acid homology with v-*erbA*, which does not bind the hormone; the v-*erbA* gene codes for a protein with 13 amino acid changes and a 9 amino acid deletion located 3 residues in from the C-terminus of c-*erbA*. These

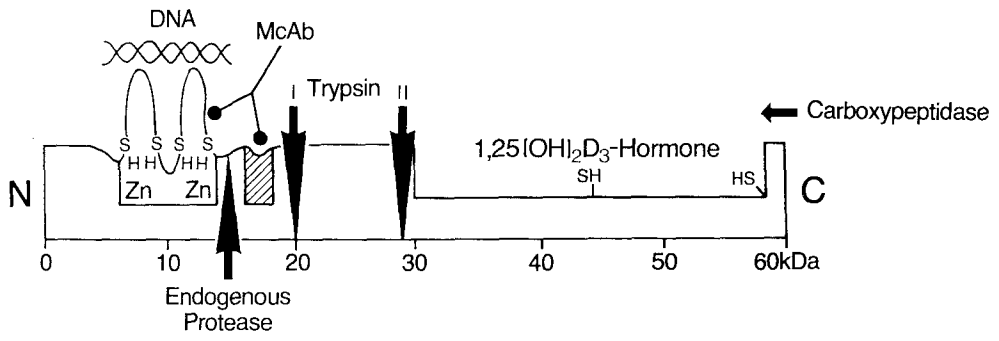


Fig. 4. Schematic Model of the Chick $1,25(OH)_2D_3$ Receptor Protein. N,C, amino- and carboxy-termini distinction, respectively; SH, sulfhydryl group of cysteine; McAb, monoclonal antibody; cross hatching, antibody determinant; arrows, enzyme cut-sites. Evidence for the presence of cysteine residues in the hormone binding domain of the intact $1,25(OH)_2D_3$ receptor (23) as well as its hormone bound, nonDNA-binding fragments (data not shown) has been obtained.

alterations in the C-terminal portion of the v-erbA protein are likely responsible for its deficiency in binding thyroid hormone.

Fig. 4 illustrates the biochemical anatomy of the $1,25(OH)_2D_3$ receptor which incorporates the data from carboxypeptidase digestion as well as from previous proteolytic mapping (3,4). Limited digestion with low (I) and high (II) concentrations of trypsin (see Fig. 4) have been shown to produce hormone bound fragments of molecular weight 40 and 30 kDa, respectively (3,4), thus delimiting the $1,25(OH)_2D_3$ binding domain to approximately 30 kDa. An endogenous protease in chick intestinal cytosol cleaves the 60 kDa receptor to a 45 kDa fragment that bound both $1,25(OH)_2D_3$ and monoclonal antibody, but not DNA (4), allowing the placement of the antigenic determinant between the hormone and DNA binding regions (Fig. 4). Also, a 20 kDa immunoreactive DNA binding fragment was recovered after limited digestion with low trypsin levels (4), indicating that the DNA binding domain was confined to the opposite end of the receptor from the hormone binding region. The DNA binding domain of the avian $1,25(OH)_2D_3$ receptor has recently been cloned (21) and shown to possess positionally conserved cysteines which are thought to coordinate zinc ions to form DNA binding fingers. This finding is consistent with the stabilization of the receptor DNA binding domain by zinc (22) and the elution of the receptor (15) and its fragments (4) from DNA with pCMBS. The present results with carboxypeptidase provide a more complete map of the receptor molecule (Fig. 4), with the

DNA binding fingers located near the N-terminus and the $1,25(OH)_2D_3$ hormone binding site in the C-terminal half of the protein. Our data also comprise the first biochemical evidence, utilizing a receptor protein, that the family of steroid and thyroid hormone receptors possess a C-terminal sequence critical to the integrity of hormone binding.

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