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24,25-Dihydroxyvitamin D_3 Binds to Catalase

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Abstract There is increasing evidence that the vitamin D metabolite, 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) has endocrine actions. In the current work, we report that an endogenous binding protein for 24,25$(OH)_2D_3$ is catalase, based on sequence analysis of the isolated protein. An antibody (Ab 365) generated against equivalent protein recognized bovine catalase and a 64 kDa band in subcellular fractions of chick intestine. A commercially available anti-catalase antibody reduced specific $[^3H]$24,25$(OH)_2D_3$ binding in subcellular fractions of chick intestine by greater than 65%, relative to the same fractions treated with an unrelated antibody (Ab 099). The same commercially available anti-catalase was able to block the inhibitory actions of 24,25$(OH)_2D_3$ on ^{32}P uptake in isolated intestinal epithelial cell suspensions. We subsequently characterized binding of steroid to commercially available catalase, and found that between 0 and 5 nM of enzyme added to subcellular fraction P_2 (20,000*g*, 10-min post-nuclear pellet) resulted in a linear increase in the amount of $[^3H]$24,25$(OH)_2D_3$ specifically bound. Additional studies indicated that 25(OH)D_3 was an effective competitor for binding, whereas 1,25$(OH)_2D_3$ only poorly displaced $[^3H]$24,25$(OH)_2D_3$. Saturation analyses with added catalase yielded a physiologically relevant affinity constant ($K_D = 5.6 \pm 2.7$ nM) and a $B_{max} = 209 \pm 34$ fmols/mg protein, comparable to previous studies using purified basal lateral membranes or vesicular fractions. Moreover, in a study on subcellular fractions isolated from chickens of varying ages, we found that in females, both specific $[^3H]$24,25$(OH)_2D_3$ binding and catalase activity increased from 7- to 58-week-old birds, whereas in males, elevated levels of both parameters were expressed in preparations of 7- and 58-week-old birds. The data suggest that signal transduction may occur through modulation of hydrogen peroxide production. J. Cell. Biochem. 97: 1259–1266, 2006. © 2005 Wiley-Liss, Inc.

Key words: vitamin D; rapid actions; chick intestine; phosphate uptake

A growing body of evidence has indicated that the vitamin D metabolite 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) has specific, physiologically significant actions that are distinct from 1,25-dihydroxyvitamin D_3 [reviewed in Larsson and Nemere, 2001; Farach-Carson and Nemere, 2003]. In the perfused duodenal loop of chick, we have found that 24,25$(OH)_2D_3$ inhibits the rapid 1,25$(OH)_2D_3$-mediated stimulation of phosphate transport [Nemere, 1996], as well as calcium transport [Nemere et al., 2002]. While the 24,25$(OH)_2D_3$ binding protein is evident on the cell surface [Nemere et al., 1994; Larsson et al., 2001], a larger pool of binding activity was found intracellularly [Nemere et al., 2002].

In concert with the reports from the white Leghorn cockerel, putative receptor proteins for 24,25$(OH)_2D_3$ in plasma membranes and evidence for physiological functions have been demonstrated in several other animal species and tissues [Seo et al., 1996; Kato et al., 1998; Pedrozo et al., 1999; Larsson et al., 2001, 2003; Boyan et al., 2002]. The putative receptors for 24,25$(OH)_2D_3$ show allosteric binding to 24,25$(OH)_2D_3$, and mediate rapid membrane-initiated responses regulating calcium homeostasis, cell differentiation and maturation, and trigger second messenger systems in

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chondrocytes, osteoblasts, and intestinal cells [Lieberherr, 1987; Caffrey and Farach-Carson, 1989; Yukihiro et al., 1994; Larsson et al., 1995, 2001, 2003; Nemere, 1996; Takeuchi and Guggini, 1996; Boyan et al., 2002; Nemere et al., 2002; Larsson and Nemere, 2003].

In the present study, we report the results of protein sequence analyses and partial characterization of the binding protein.

MATERIALS AND METHODS

Animals, Surgical Procedures, and Subcellular Fractionation

All protocols were approved by the Institutional Animal Care and Use Committee at Utah State University. White Leghorn cockerels or pullets were obtained on the day of hatch and raised for 7, 14, 28, and 58 weeks as previously described [Larsson and Nemere, 2003]. On the day of use, birds were anesthetized with chloropent (0.3 ml/100 g body weight), the duodenal loop surgically removed to ice-cold saline and chilled for 15 min. The pancreas was removed, the duodenal segment slit longitudinally, and rinsed in ice-cold saline prior to collection of mucosa by scraping into 40 ml of homogenization medium (250 mM sucrose, 5 mM histidine-imidazole, 2 mM EGTA, pH 7.0) [Nemere et al., 1994]. After disruption in a Potter–Elvejem homogenizer, the post-nuclear supernatant (1,000*g*, 20 min, 4°C) was further centrifuged at 20,000*g*, 10 min (4°C) to yield P_2. In some studies, P_2 was further resolved on Percoll gradients [Nemere, 1996] and fractions 1–3 collected for binding- and enzyme-activity studies.

Cell Isolation and Incubation Protocols

Cells were isolated by citrate chelation as previously described [Nemere et al., 2004; Sterling and Nemere, 2005] and collected by low speed centrifugation at 500*g*, 5 min (4°C). The pellets were resuspended in 40 ml of room temperature Gey's Balanced Salt Solution (GBSS) lacking bicarbonate [Sterling and Nemere, 2005]. Fourteen milliliters of cell suspension were added to 28 μCi of $H_3{}^{32}PO_4$ (PerkinElmer Life Sciences, Boston, MA) with mixing, and further divided into four 3.2-ml aliquots. Three of the aliquots were combined with anti-catalase (LabFrontiers, Seoul, South Korea; 1/2,000, final dilution). After removing 100 μl baseline samples at T = −5 and −1 min, the remaining suspensions were treated with either vehicle or steroids. The four incubation conditions were controls (0.05% ethanol, final concentration) plus anti-catalase, 300 pM $1,25(OH)_2D_3$ plus anti-catalase, 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus anti-catalase, and 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$. Additional samples were taken at T = 1, 3, 5, 7, and 10 min. Each sample was pipetted into 900 μl of ice-cold GBSS and centrifuged at 1,000*g*, 5 min (4°C). Supernatants were decanted, and the inside of the tubes swabbed while still in the inverted position. Each pellet was dispersed in 500 μl of reagent grade water and analyzed for protein and radioactivity. For each sample in the treated phase, cpm/μg of protein was normalized to the average basal cpm/μg of protein.

Chromatography

The pellet fraction (P_2) containing basal lateral membranes and intracellular organelles [Nemere, 1996] was further homogenized in 10 mM Tris, 2 mM EDTA, 1 mM dithiothreitol, pH 7.4 (TED) to extract the binding activity, and the high speed supernatant fractions (105,000*g*, 1 h, 4°C) filtered using Centricon Plus-20 (Fisher Scientific, Dallas, TX) centrifugal filtration devices prior to anion exchange chromatography [Nemere et al., 2002] on a Mono Q HR 5 column (Amersham Pharmacia, Arlington Heights, IL). Eluted fractions were tested for specific $[^3H]24,25(OH)_2D_3$ binding (see below), and the peak fractions pooled for further separation by gel filtration [Nemere et al., 2002] on a Superose 12 column (Amersham Pharmacia). The eluted fractions were again tested for specific binding, and peak fractions pooled and lyophilized.

SDS–PAGE, Sequence Analysis, and Antibody Production

The lyophilized protein was dissolved in sample buffer and resolved on 8% polyacrylamide gel, followed by staining with Coomassie Brillant Blue. A major band of 60–64 kDa was submitted to the Center for Integrated BioSystems (CIB) at Utah State University for tryptic digestion and sequencing by Edman degradation. Two equivalent bands in adjacent lanes of the gel were excised and used to immunize rabbits. Polyclonal antisera (Ab 365 and Ab 377) were generated and collected by CIB staff.

Western Analysis

Samples of commercially available bovine catalase (Sigma Chemical Co., St. Louis, MO) and P_2 fractions were resolved on SDS–PAGE and blotted onto PVDF membranes [Larsson and Nemere, 2003]. After blocking and washing [Larsson and Nemere, 2003], the PVDF membrane was incubated with primary antibody (Ab 365) at a 1/200 dilution. Subsequent steps were as previously described [Larsson and Nemere, 2003].

Binding of $[^3H]24,25(OH)_2D_3$

Fractions were generally incubated at a level of 100 μg protein and 16 nM $[^3H]24,25(OH)_2D_3$ unless otherwise indicated. Total binding was performed in triplicate and nonspecific binding (in the presence of a 200-fold molar excess of unlabeled metabolite) in duplicate as previously described [Nemere et al., 2002]. Following an overnight incubation on ice, bound and free radioactivities were separated by the hydroxylapatite (HAP) assay [Larsson and Nemere, 2003]. Protein bound to HAP was washed three times with 0.5% Triton in TED, and radioactivity eluted with ethanol. The ethanol was evaporated prior to addition of liquid scintillation cocktail and determination of radioactivity.

Analytical Determinations

Catalase activity was determined spectrophotometrically by the method of Aebi [1984]. Protein was assessed using the Bradford reagent (BioRad, Hercules, CA) against bovine γ-globulin as standard. Radioactivity was determined by liquid scintillation spectrophotometry.

RESULTS

Sequence Analysis

Tryptic digestion and sequence determination by the Edman degradation procedure produced five peptides corresponding to the internal sequence of a mutant catalase (accession number AY040626.1). Figure 1 provides the 527 amino acid sequence with the tryptic peptides indicated in bold and underlined. Western analysis was performed using Ab 365 as primary antibody against samples of bovine catalase and P_2 fractions prepared from chick intestine. As shown in Figure 2, Ab 365, directed against a protein equivalent to that which was sequenced, recognized both commercially available catalase, as well as a protein in P_2 fractions.

Effect of Anti-Catalase Antibody on $[^3H]24,25(OH)_2D_3$ Binding in Subcellular Fractions and on Cellular Response to Steroids

Percoll gradient fractions 1 and 2 were prepared and mixed either with Ab 099 (against the $1,25D_3$-MARRS protein) at a 1/200 dilution or anti-catalase antibody at a 1/2,000 dilution. Aliquots were then taken to assess specific binding of $[^3H]24,25(OH)_2D_3$. As illustrated in Figure 3, anti-catalase antibody reduced specific binding greater than 65%, relative to equivalent fractions pre-incubated with Ab 099.

Using an equivalent dilution of anti-catalase antibody, we tested whether it could neutralize

1 msdsrdpasd qmkqwkeqra sqrpdvlttg ggnpigdkln imtagsrgpl lvqdvvftde
61 mahfdrerip ervvhakgag afgyfevthd itryskakvf ehigk**rtpia vr**fstvages
121 gsadtvrdpr gfavkfyted gnwdlvgnnt piffirdail fpsfihsqkr npqthlkdpd
181 mvwdfwslrp eslhqvsflf sdrgipdghr hmngygshtf klvnadgeav yckfhyktdq
241 giknlpvgea g**rlaqedpdy glr**dlfnaia ngnypswtfy iqvmtfkeae t**fpfnpfdlt**
301 **k**vwphkdypl ipvgklvlnk npvnyfaeve qmafdpsnmp pgiepspdkm lqg**rlfaypd**
361 **thr**hrlgpny lqipvncpyr arvanyqrdg pmcmhdnqgg apnyypnsfs apeqqrsale
421 hsvqcavdvk rfnsanedsv tqvrtfytkv lneeerkrlc eniaghlkda qlfiqkkav**k**
481 **nftdvhpdyg ar**iqalldky naekpknaih tytqagshma akgkanl

Fig. 1. Tryptic peptides of the $24,25(OH)_2D_3$ binding protein identified in chick (equivalent to mutant mouse catalase, AY040626.1). Chick mucosal fraction P_2 (containing intracellular organelles and basal lateral membranes) was extracted by homogenization with 10 mM Tris, 2 mM EDTA, 1 mM dithiothreitol, pH 7.4; TED, subjected to high-speed centrifugation (100,000*g*, 1 h, 4°C), and filtered prior to chromatography on an anion exchange column. Fractions eluted with a linear 0–1 M NaCl gradient were analyzed for specific $[^3H]24,25(OH)_2D_3$ binding (see below). Fractions with the highest specific binding activity were pooled, subjected to gel filtration chromatography, and the fractions again analyzed for specific binding. Those eluting at 50–60 kDa were pooled and lyophilized until further use. The lyophilized material was dissolved in SDS–PAGE sample buffer resolved by electrophoresis, and the greatly enriched band of appropriate molecular weight excised for tryptic digestion and sequencing by Edman degradation. Amino acid sequences in bold and underlined, correspond to peptide sequences obtained from the chick protein.

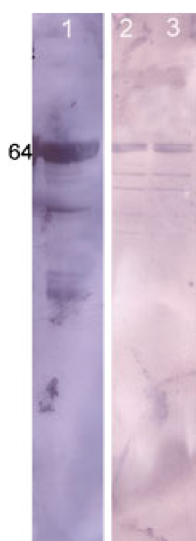

Fig. 2. Western analysis with Ab 365. Protein from the SDS–PAGE described in the legend to Figure 1 was excised and used to generate a polyclonal Ab 365, subsequently used in Western analysis of 12.5 μg of bovine catalase (**lane 1**), and two independent preparations of chick intestinal fraction P_2 (20,000*g* post-nuclear pellet; **lanes 2**, **3**, each 25 μg of protein). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

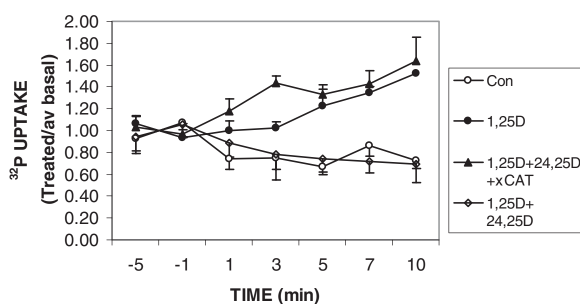

Fig. 4. Anti-catalase neutralization of the 24,25$(OH)_2D_3$ inhibitory effect on ^{32}P uptake. Intestinal epithelial cells were isolated by citrate chelation and resuspended in GBSS, and a portion mixed with ^{32}P to give 2 μCi/ml. Cells were then treated as follows: (1) Controls were exposed to anti-catalase (1/2,000 final dilution) at T = −10 min, and vehicle (0.05% ethanol, final concentration) at T = 0; (2) exposed to anti-catalase (1/2,000 final dilution) at T = −10 min and 300 pM 1,25$(OH)_2D_3$ at T = 0; (3) exposed to anti-catalase (1/2,000 final dilution) at T = −10 min and 300 pM 1,25$(OH)_2D_3$ in combination with 6.5 nM 24,25$(OH)_2D_3$ at T = 0; (4) the steroid combination without pretreatment with antibody. Samples (100 μl) were taken at the indicated times and pipetted into ice-cold GBSS, then centrifuged at 1,000*g*, 5 min, 4°C. The supernatants were decanted, the insides of the tubes swabbed, and the pellets resuspended in water for determination of radioactivity and protein. Values represent mean ± SEM for four independent experiments.

the inhibitory effect of 24,25$(OH)_2D_3$ on 1,25$(OH)_2D_3$-stimulated phosphate uptake. As shown in Figure 4, 300 pM 1,25$(OH)_2D_3$ enhanced cellular ^{32}P uptake to 200% of controls after 10 min of treatment ($P < 0.05$), while co-incubation of cells with both steroids resulted in ^{32}P levels equivalent to controls. However, inclusion of anti-catalase antibody effectively negated 24,25$(OH)_2D_3$-induced inhibition, resulting after 10 min in ^{32}P levels that were 230% of uptake in cells incubated with both steroids but without antibody ($P < 0.05$; Fig. 4).

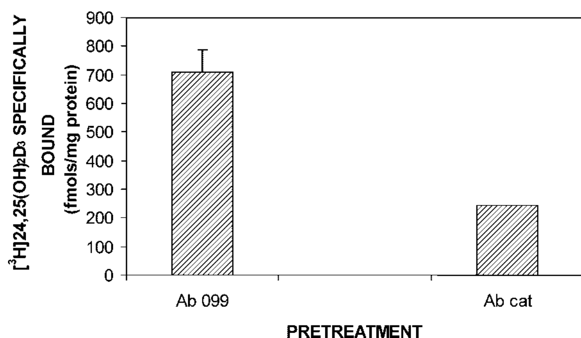

Fig. 3. Anti-catalase antibody inhibits specific binding of $[^3H]$24,25$(OH)_2D_3$. Post-nuclear P_2 was further separated on Percoll gradients, fractions 1 and 2 collected and pooled. Aliquots were mixed with TED and either Ab 099 (to the 1,25D_3-MARRS protein) at a 1/200 dilution, or anti-catalase at a 1/2,000 dilution (Ab cat). Samples were incubated (overnight on ice) in triplicate with 16 nM $[^3H]$24,25$(OH)_2D_3$ (total binding) and in duplicate with radioactively labeled steroid in the presence of a 200-fold molar excess of unlabeled metabolite (nonspecific binding). The following morning, bound and free steroid were separated with hydroxylapatite (HAP), washed with 0.5% Triton X-100 in TED, and steroid eluted with ethanol. Ethanol was evaporated prior to liquid scintillation counting. Values represent average ± range for two independent experiments.

Binding of $[^3H]$24,25$(OH)_2D_3$ to Catalase

To further characterize binding, known quantities of commercially available catalase monomer were added to P_2 pellet fractions (which provided nonspecific binding components) and specific binding of $[^3H]$24,25$(OH)_2D_3$ determined. As shown in Figure 5, specific binding of $[^3H]$24,25$(OH)_2D_3$ increased with catalase addition, and was essentially linear between 0 and 5 nM catalase monomer added to P_2 ($R^2 = 0.95$).

Additional studies were conducted with 1 nM catalase added to P_2. Figure 6 illustrates the results of competition studies, in which specific binding in incubations with 16 nM $[^3H]$24,25$(OH)_2D_3$ was set to 100%. Incubation of 1 nM catalase in P_2 and 3,200 nM unlabeled 25$(OH)D_3$ resulted in essentially complete competition, whereas 3,200 nM unlabeled 1,25$(OH)_2D_3$ yielded only 40% competition (Fig. 6).

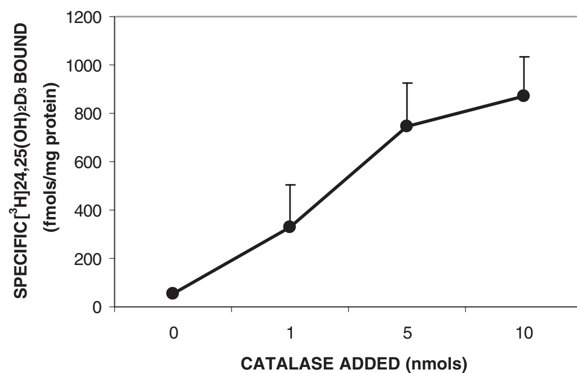

Fig. 5. Specific binding of [^{3}H]24,25(OH)$_2$D$_3$ to increasing concentrations of catalase. Commercially available enzyme was added to P$_2$ fractions (100 μg of protein). Binding was determined as described in the legend to Figure 3 and found to be linear ($R^2 = 0.95$) between 0 and 5 nM of added catalase. Values represent mean ± SEM for six independent determinations.

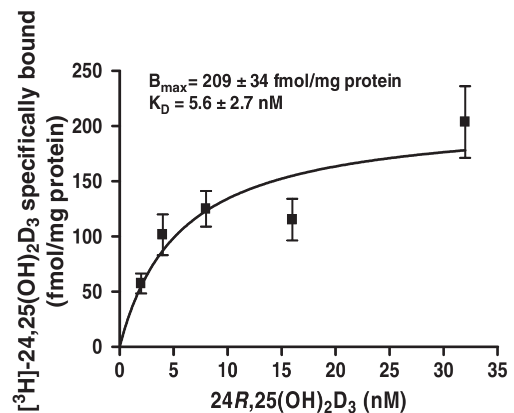

Fig. 7. Saturation analyses of 1 nM catalase added to P$_2$. Samples were incubated with increasing concentrations of [^{3}H]24,25(OH)$_2$D$_3$ in the absence or presence of a 200-fold excess of unlabeled steroid, and processed as described in the legend to Figure 3. Values represent mean ± SEM for three independent experiments.

Saturation analyses were then performed using 1 nM catalase monomer added to P$_2$. Results of these studies indicated that binding was saturable (Fig. 7). Under the conditions used, the results could be fitted to a rectangular hyperbola, with K_D calculated as 5.6 nM and $B_{max} = 209$ fmol/mg protein (Fig. 7).

Comparison of Catalase Activity In Vivo With Specific [^{3}H]24,25(OH)$_2$D$_3$ Binding as a Function of Age

Previous work had indicated that the highest levels of specific [^{3}H]24,25(OH)$_2$D$_3$ binding in subcellular fractions of chick intestinal mucosa were observed in the densest fractions 1 and 2, with decreased binding in fraction 3 [Nemere et al., 2002]. This was also found to be the case in female chickens of ages 7, 14, 28, and 58 weeks of age (Fig. 8, top panel). However, there was also a striking increase in specific binding activity with age: in fractions 1 and 2, an approximate four- to fivefold increase occurred between 7 and 58 weeks of age (Fig. 8, top panel). Catalase specific activity roughly paralleled binding activity (Fig. 8, bottom panel). Catalase specific activity did not decline as precipitously in fraction 3 as did metabolite binding; however, an age-dependent increase in the peroxisomal marker enzyme was observed in that catalytic activity was lowest in fractions prepared from mucosa of 7-week-old female chicks, increasing nearly twofold in preparations from 28- to 58-week-old birds.

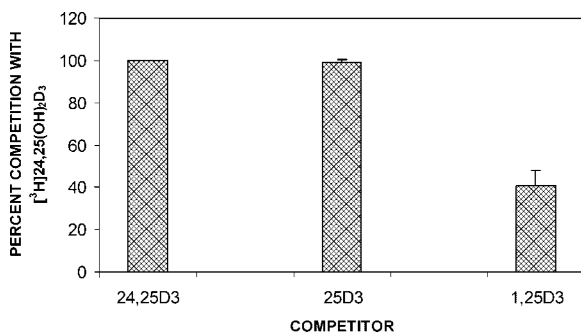

Fig. 6. Analyses of binding specificity. Fractions of P$_2$ containing 1 nM of catalase were incubated with 16 nM [^{3}H]24,25(OH)$_2$D$_3$ in triplicate in the absence of competitor, and in triplicate in the presence of a 200-fold molar excess of either 24,25(OH)$_2$D$_3$, 25(OH)D$_3$, or 1,25(OH)$_2$D$_3$. After overnight incubation on ice, bound and free steroids were separated by HAP as described in the legend to Figure 3. Results represent mean ± SEM for six independent experiments.

Similar studies were conducted with fractions prepared from 7- to 58-week-old male birds, representing the two extremes in ages. Again, specific [^{3}H]24,25(OH)$_2$D$_3$ binding was higher in fractions 1 and 2 of Percoll gradients (Fig. 9, top panel). However, these fractions exhibited elevated levels of binding in preparations from duodenal mucosa of 7-week-old chicks, compared to equivalent preparations from female chicks (compare Figs. 8 and 9, top panels). Catalase specific activities in preparations from male birds likewise did not show an age-related difference (Fig. 9, bottom panel), but were also quite elevated relative to preparations from female chickens (compare with Fig. 8, bottom

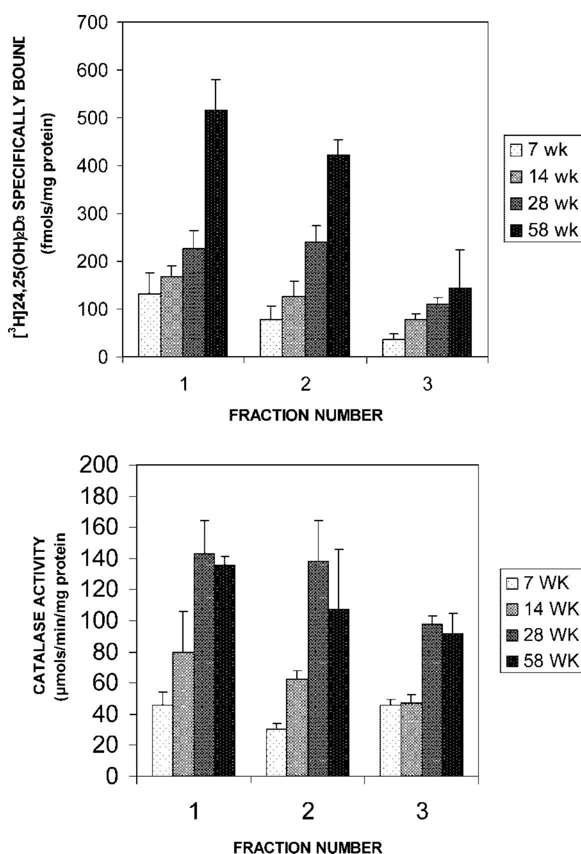

Fig. 8. Age-dependent increase in specific [^{3}H]24,25$(OH)_2D_3$ binding and catalase activity in organelles prepared from female chickens. Female chickens were raised for 7, 14, 28, or 58 weeks prior to collection of intestinal mucosa for subcellular fractionation. P_2 fractions were further resolved on Percoll gradients, and fractions 1–3 analyzed for specific binding (**upper panel**) and catalase specific activity (**lower panel**), the latter by the decrease in the absorbance of 20 mM H_2O_2 at 240 nM. Values represent mean ± SEM of three independent experiments.

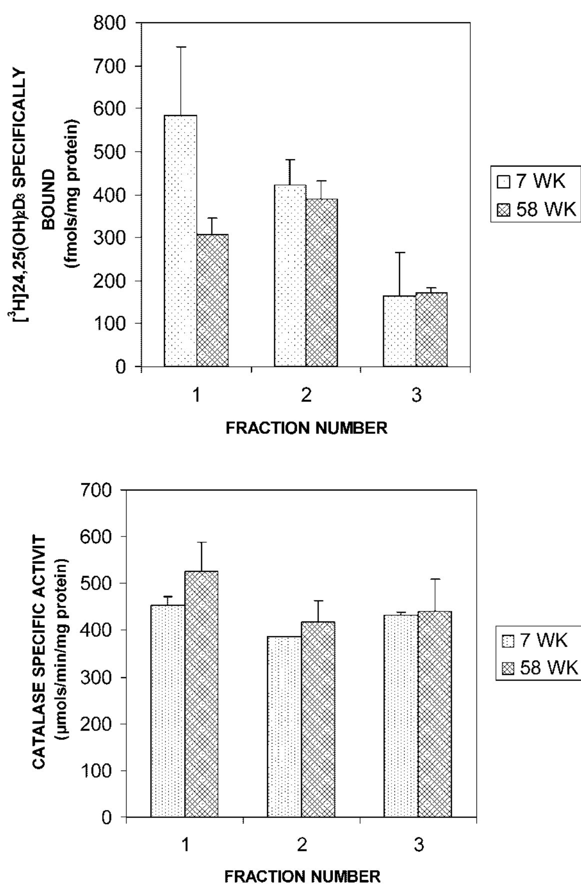

Fig. 9. Lack of an age-dependent effect on specific [^{3}H]24,25$(OH)_2D_3$ binding and catalase activity in organelles prepared from male chickens. Percoll gradient fractions were prepared from P_2 of intestinal mucosa obtained from 7- and 58-week-old male chickens. Fractions 1–3 were assayed for specific [^{3}H]24,25$(OH)_2D_3$ binding (**upper panel**) as described in the legend for Figure 3 and catalase specific activity, as described in the legend to Figure 8. Values represent mean ± SEM of three independent experiments.

panel). Thus, preparations from female birds exhibited an age-dependent increase in both specific [^{3}H]24,25$(OH)_2D_3$ binding and catalase activity with age, whereas equivalent preparations from duodenal mucosa of male birds exhibited quite high levels of both metabolite binding and catalase activity regardless of age.

DISCUSSION

The present work identifies and partially characterizes a binding protein for the vitamin D metabolite, 24,25$(OH)_2D_3$, which shares sequence homology with a mutant catalase described in mouse [Wang et al., 2001]. It is not known whether the [^{3}H]24,25$(OH)_2D_3$ binding protein identified in chick duodenal mucosa has catalytic activity or not: Wang et al. [2001] found reduced activity due to a point mutation in which Asp at position 439 was changed to Ser; the mutation reduced activity and interfered with tetramerization.

Studies using added bovine catalase demonstrated that addition of 1 nM increased specific [^{3}H]24,25$(OH)_2D_3$ binding by somewhat less than 0.5 pM. The lack of 1:1 stoichiometry could be due to lack of tetramerization, either attributable to effects of prior lyophilization, or interaction of the monomer with other cellular components. Both possibilities have merit. In competition studies, a large molar excess of 1,25$(OH)_2D_3$ only weakly displaced binding of [^{3}H]24,25$(OH)_2D_3$, as previously reported [Nemere et al., 2002]. In contrast, 25$(OH)D_3$

yielded complete competition in the current study, yet was less effective with native preparations [Nemere et al., 2002]. In a physiological context, both $24,25(OH)_2D_3$ and $25(OH)D_3$ have been found to inhibit the rapid $1,25(OH)_2D_3$-mediated stimulation of intestinal phosphate transport [Nemere, 1996].

Saturation analyses with exogenous catalase yielded an affinity comparable to that observed for native preparations of dense gradient fractions and purified basal lateral membranes [Nemere et al., 2002], which moreover are in agreement with values obtained for carp and cod basal lateral membranes [Larsson et al., 2001]. The number of binding sites determined in the present work is also comparable to earlier reports [Larsson et al., 2001; Nemere et al., 2002]. Perhaps a significant difference is that we did not detect cooperativity in the present analyses, whereas positive cooperativity was previously observed in basal lateral membranes, and negative cooperativity was found in intracellular organelle preparations [Nemere et al., 2002]. During the purification process, it was observed that binding activity eluted with a wide range of salt concentrations during anion exchange chromatography, but yielded a single molecular weight peak on gel filtration. Thus, the site-specific, cooperative binding properties observed with different subcellular fractions may be due to interaction with heterologous components. In this context, it should be noted that catalase has been found tightly bound to the cell surface in other tissues [Watanabe et al., 2003]. Thus, catalase may act as the membrane recognition moiety that subsequently directs the steroid to intracellular organelles [Nemere et al., 2002]. This is supported by the findings that (1) anti-catalase antibody inhibits $[^3H]24,25(OH)_2D_3$ binding to vesicular fractions in vitro, and (2) addition of the same antibody to intact intestinal epithelial cells abrogates the inhibitory effects of $24,25(OH)_2D_3$ on $1,25(OH)_2D_3$-stimulated phosphate uptake.

Our observations on binding activity versus catalase activity in vivo further support the identification of the peroxisomal enzyme as an endogenous binding protein for $24,25(OH)_2D_3$. In female chickens, both specific $[^3H]24,25(OH)_2D_3$ binding and enzyme activity increased with age, whereas in males, both activities were elevated regardless of age. The incomplete correlation between catalase activity and binding of metabolite could be attributed to either differences in isozyme content, or the presence of a mutant catalase.

Finally, identification of catalase as an endogenous $24,25(OH)_2D_3$ binding protein suggests a mechanism for signal transduction. The peroxisomal marker enzyme is best known for its ability to remove hydrogen peroxide. Hydrogen peroxide, in turn, has recently been implicated as a newly found signal transducer [Waypa et al., 2002; Yano and Yano, 2002; Watanabe et al., 2003; Wood et al., 2003]. Studies to address this possibility are under way.

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