Biochemical characterization and purification of a binding protein for 24,25-dihydroxyvitamin D₃ from chick intestine

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Abstract

An earlier study revealed that 24R,25-dihydroxyvitamin D₃ (24R,25(OH)₂D₃) inhibits the rapid actions of 1,25(OH)₂D₃ on stimulation of calcium transport in perfused duodena, as well as activation of protein kinases C and A. In the present work, a specific binding protein (24,25-BP) has been identified and partially characterized. Percoll-gradient resolution of differential centrifugation fractions from mucosal homogenates revealed the highest levels of specific [³H]24R,25(OH)₂D₃ binding to be in lysosomes (approximately eight to tenfold greater than in basal lateral membrane fractions). Incubation of isolated enterocytes with 6.5 nM [³H]24R,25(OH)₂D₃ for 10 s also demonstrated targeting of the steroid to lysosomal fractions. Using freshly isolated lysosomal fractions, time course studies indicated maximal specific binding after a 2-h incubation on ice. Western analyses revealed that the serum transport protein, DBP (vitamin D binding protein), was absent from both lysosomal and basal lateral membrane fractions. Protein dependence studies demonstrated linear binding between 0.05 and 0.155 mg of lysosomal protein. Saturation analyses yielded $K_d = 7.4 \pm 1.8$ nM, $B_{max} = 142 \pm 16$ fmol/mg protein for lysosomes, and $K_d = 8.5$ nM, $B_{max} = 149 \pm 25$ fmol/mg protein for

basal lateral membranes. Hill analyses of lysosomal binding yielded a Hill coefficient of 0.57 ± 0.11 , indicative of negative cooperativity. Studies with lysosomal proteins revealed a $81\% \pm 7\%$ competition of 24S,25(OH)₂D₃ with [³H]24R,25(OH)₂D₃ for binding ($P > 0.05$, relative to competition with 24R,25(OH)₂D₃), while 25(OH)D₃ and 1,25(OH)₂D₃ yielded $53\% \pm 13\%$ and $39\% \pm 11\%$ competition respectively (each, $P < 0.05$, relative to competition with 24R,25(OH)₂D₃). The apparent affinity of 24S,25(OH)₂D₃ for 24,25-BP led to testing of the metabolites effectiveness in the perfused duodenal loop system. Vascular perfusion with 130 pM 1,25(OH)₂D₃ stimulated ⁴⁵Ca transport to 2.5-fold above control levels after 40 min, while simultaneous perfusion with 6.5 nM 24S,25(OH)₂D₃ and 130 pM 1,25(OH)₂D₃ abolished the stimulatory activity completely. Purification of the 24,25-BP by chromatography revealed a single protein band upon SDS-PAGE and silver staining of 66 kDa. The combined results suggest that 24R,25(OH)₂D₃ may mediate its hormonal activities through a specific binding protein.

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Introduction

Vitamin D is metabolized in the liver to 25-hydroxyvitamin D₃, and in the kidney to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃). 1,25(OH)₂D₃ is well known for its hormonal activity in diverse cell types, and its ability to enhance intestinal calcium and phosphate absorption. 24,25(OH)₂D₃ has not been as widely studied, perhaps because of the perception that it is an inactivation product. However, a growing body of literature has indicated that 24,25(OH)₂D₃ has important effects in development (Henry & Norman 1978), bone (Sömjen *et al.* 1983, Lidor *et al.* 1987, Schwartz *et al.* 1988, Swain

et al. 1993, Khoury *et al.* 1995, Yukihiro *et al.* 1994, Takeuchi & Guggino 1996, Ono *et al.* 1996, Seo & Norman 1996, 1997, Kato *et al.* 1998), and parathyroids (Carpenter *et al.* 1996). More recently (Nemere 1996a, 1999), it has been demonstrated that in chick intestine, 24R,25(OH)₂D₃ inhibits the rapid stimulation of calcium transport mediated by 1,25(OH)₂D₃. The ability to block intestinal calcium transport further appears to be an important survival mechanism in marine fish (Sundell & Björnsson 1990, Larsson *et al.* 1995, Larsson 1999). The ability of 24R,25(OH)₂D₃ to inhibit 1,25(OH)₂D₃-enhanced protein kinases A and C activities in chick intestine (Nemere 1999) also suggests that a specific binding protein may exist for 24,25(OH)₂D₃ that has

receptor-like properties. The current report describes a candidate protein.

Materials and Methods

Animals

White leghorn cockerels (Merrill Poultry, Poul, ID, USA) were raised for 4–7 weeks on a vitamin D-replete diet (Nemere 1996a). All animal procedures were approved by the Institutional Animal Care and Use Committee.

Subcellular fractionation

On the day of use, chicks were anesthetized, the duodenal loop surgically removed to ice-cold saline and chilled for 15 min. After removal of the pancreas, the segment was slit longitudinally, placed on an inverted Petri dish on ice, and the mucosa collected by scraping with a microscope slide. Scrapings from two duodena were homogenized in 40 ml of 250 mM sucrose, 5 mM histidine–imidazole, 1 mM EGTA, pH 7.0, as previously described (Nemere 1996b). Differential centrifugation yielded pellets P₁ (1000 g, 20 min; containing nuclei and brush borders), P₂ (20 000 g, 20 min, containing lysosomes, mitochondria, Golgi and basal lateral membranes) and P₃ (105 000 g, 1 h, containing microsomes). Fractions P₂ and P₃ were further resolved on Percoll (Amersham Pharmacia, Piscataway, NJ, USA) gradients (Nemere 1996b), and 18, 52-drop fractions collected. Earlier reports (Nemere *et al.* 1986, Nemere 1996b) described characterization of the fractions by marker enzyme analyses, fold enrichment, and percent recovery. From the bottom of the gradient, fractions 1–3 were identified as lysosomes, fractions 4–13 as mitochondria, fractions 14–15 Golgi, and fractions 16–18 as basal lateral membranes (Nemere *et al.* 1986, Nemere 1996b). In the current work protein was determined with the Bradford reagent (BioRad, Hercules, CA, USA) against bovine gamma globulin as standard (Sigma Chemical Co., St Louis, MO, USA).

Binding of [³H]24R,25(OH)₂D₃ to isolated fractions

For each sample, five tubes were prepared to contain [³H]24R,25(OH)₂D₃ (76 Ci/mmol) in the absence (total binding, three tubes) or presence of a 200-fold molar excess of unlabeled competitor (nonspecific binding, two tubes) in 20 µl of ethanol. Each tube then received TED buffer (10 mM Tris, 1.5 mM EDTA, 2 mM dithiothreitol, pH 7.4) and sample. Incubations were conducted on ice and bound and free metabolites separated by hydroxyl-apatite (HAP) assay (Nemere *et al.* 1994). The radioactivity extracted from the final HAP pellet was dried in glass liquid scintillation vials. Six milliliters of scintillation cocktail were then added and the sample counted in a Beckman LS6500 counter. For saturation analyses specific 24R,25(OH)₂D₃ binding to membranes was tested by

nonlinear regression. The concentration of the labeled hormone was plotted against the amount of specifically bound, labeled hormone (fmol/mg protein), and the data were fitted to a three-parameter sigmoid function:

$$f(x) = a / (1 + e^{-(x - x_0/b)})$$

The coefficient of variation (R^2) and the adjusted coefficient of variation ($\text{adj}R^2$) were used as a measure of how well the regression model described the data in the saturation and Hill analyses (Altman 1991). A one-way ANOVA with *F* statistics was used to gauge the contribution of the independent variable to predict the dependent variable (Altman 1991). A $P < 0.05$ was considered as statistically significant.

A one-way ANOVA followed by a Student–Newman–Keuls *post hoc* test was used to test for significant differences between groups in the metabolite competition studies. The testing was two-tailed and the significance level was set to $P < 0.05$. Data are presented as means $\pm$ S.E.M.

[³H]24R,25(OH)₂D₃ uptake by isolated intestinal cells

After removal of the pancreas from each of two duodenal loops, segments were everted, rinsed in 0.9% saline and then placed in citrate chelation solution (Nemere & Campbell 2000). Cells from the first two 15-min incubations were pooled into two 50 ml polypropylene tubes and collected by centrifugation (500 g, 5 min, 4 °C). The supernatants were decanted, and while still in the inverted position, the insides of the tubes were swabbed with a Kimwipe. The pellets were gently resuspended with a Teflon rod in 10 ml of Gey's Balanced Salt Solution (GBSS) containing 0.1% BSA. The cell suspension was then rapidly combined with an equivalent volume of GBSS–BSA containing [³H]24R,25(OH)₂D₃ to give a final concentration of 6.5 nM steroid. The cells were swirled for 10 s and then collected by low speed centrifugation for homogenization and subcellular fractionation as described above.

Western analyses

SDS-PAGE was performed and proteins blotted onto PVDF membranes as described elsewhere (Jia & Nemere 1999, Nemere *et al.* 2000). Human vitamin D binding protein (DBP) was purchased from Sigma and goat anti-DBP from Incstar Corp. (Stillwater, MN, USA) and used at a dilution of 1/500. Alkaline phosphatase conjugated rabbit anti-goat antibody (1/30 000 dilution) was from Sigma.

Perfusion of duodenal loops

Procedures were as described (Nemere 1996a) for vascular perfusion of three duodena with control medium during the treated phase, three with 130 pM 1,25(OH)₂D₃,

and three with 130 pM 1,25(OH)₂D₃ plus 6.5 nM 24S,25(OH)₂D₃ during the treated phase.

Protein purification

Lysosomal fractions were collected and frozen. They were subsequently homogenized in ten volumes of cold TED buffer (25 strokes on ice) and Percoll removed by centrifugation. The supernatant fractions were passed through 0.45 µm syringe filters and then concentrated in Centrifuplus spin columns (Amicon, Beverly, MA, USA) with a 50 kDa pore size. Both the filtrate and retentate were tested for specific [³H]24,25(OH)₂D₃ binding. Protease inhibitors (Sigma) were added and the retentates stored at -20 °C. Concentrated aliquots of protein were injected onto a Mono Q HR5 anion exchange column (Amersham Pharmacia) with a 1 ml loop. Chromatography was accomplished using the Pharmacia FPLC system with a flow rate of 1 ml/min. The column was washed for 7 min with buffer A (20 mM Tris, 1 mM EDTA, pH 8.0) and then eluted with a linear gradient formed with buffer B (20 mM Tris, 1 mM EDTA, 1 M NaCl, pH 8.0). The column was then washed an additional 5 min with high salt buffer before re-equilibration in starting buffer. Aliquots of each fraction were assayed for specific binding. Those highest in specific binding were pooled, concentrated in spin columns and subjected to gel exclusion chromatography on a Superose 12 column (Amersham Pharmacia). After filtration, sample was injected with a 200 µl loop and the column eluted (0.5 ml/min) with 150 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0. Aliquots (20 µl) were taken of each sample for determination of specific [³H]24,25(OH)₂D₃ binding and SDS-PAGE followed by silver staining (Nemere *et al.* 1994).

Results

Binding of [³H]24R,25(OH)₂D₃ to isolated fractions

Mucosal scrapings were homogenized, subjected to differential centrifugation and the particulate fractions analyzed for specific [³H]24R,25(OH)₂D₃ binding. Expressed as average fmol/mg protein ± range for whole homogenates, P₁, P₂ and P₃, values were 96 ± 17, 152 ± 33, 237 ± 33 and 239 ± 63 respectively. Pellet fractions P₂ or P₃ were subsequently further resolved on Percoll gradients and analyzed for specific [³H]24R,25(OH)₂D₃ binding. Figure 1 illustrates the results of replicate experiments in which a marked enrichment of specific binding was observed in lysosomal fractions 1–2 of P₂ Percoll gradients, while no fractions in P₃ exhibited a similar level of enrichment.

[³H]24R,25(OH)₂D₃ uptake by isolated intestinal cells

The results of binding studies with subcellular fractions suggested incubation of intact isolated intestinal cells with 6.5 nM [³H]24R,25(OH)₂D₃, to determine if the cellular

Figure 1 Specific [³H]24R,25(OH)₂D₃ binding to Percoll gradient fractions prepared from post-nuclear pellets P₂ (20 000 g, 10 min) and P₃ (105 000 g, 1 h). Duodenal loops (four per experiment) were surgically removed from anesthetized chicks, and held in physiological saline for 15 min. The pancreas was then removed, the segment slit lengthwise, and the mucosae collected by scraping into homogenization buffer (250 mM sucrose, 5 mM histidine-imidazole, 1 mM EGTA, pH 7.0), 40 ml/two duodena. Unless otherwise indicated, all steps were performed at 0–4 °C. The tissue was disrupted in a Potter–Elvehjem homogenizer (70 strokes), distributed into two 50 ml polypropylene centrifuge tubes, and the volume brought to 35 ml per tube. The homogenate was centrifuged at 1000 g, 20 min to yield P₁ containing nuclei and brush borders, and the supernatant further centrifuged (20 000 g, 10 min) to yield P₂. Supernatant fraction S₂ was centrifuged (105 000 g, 1 h) to yield P₃ and S₃. Pellets were resuspended in Percoll in homogenization medium, layered over 2 ml cushions of 2.4 M sucrose, and centrifuged in a 60 Ti rotor at 20 400 r.p.m. Eighteen, 52-drop fractions were collected, and each fraction assayed for specific binding and protein. Each fraction was assayed in triplicate for total binding (16 nM [³H]24R,25(OH)₂D₃) and in duplicate in the presence of a 200-fold excess of unlabeled seco-steroid to determine nonspecific binding. After pipetting 20 µl of steroids in ethanol, 100 µl of TED were added, followed by 100 µl of sample. After mixing and an overnight incubation on ice, bound and free steroid were separated by the addition of 200 µl of HAP and three washes (0.5% Triton X-100 in TED) of the HAP pellets. Steroid was extracted from the final pellet by the addition of 1 ml of ethanol, which was decanted into scintillation vials, dried, and combined with fluor for liquid scintillation spectrophotometry. Fractions 1–3 of P₂ (lysosomes), 4–13 (mitochondria), 14–15 (Golgi) and 16–18 (basal lateral membranes) were identified in a previous series of experiments by marker enzyme analyses. Results represent the average between two independent experiments ± range.

sorting machinery directed the steroid to the same compartment. As shown in Fig. 2, a 10-s exposure to 6.5 nM radioactive seco-steroid (final concentration), followed by subcellular fractionation protocols, resulted in the highest levels of total [³H]24R,25(OH)₂D₃ localized in lysosomal fractions 1 and 2. Nonspecific binding could not be assessed in these experiments, since the level of unlabeled steroid would be supra-physiological.

One interpretation of the subcellular localization is that the binding activity resulted from the serum DBP adhering to the plasma membrane of the intestinal cells,

Figure 2 Incubation of isolated enterocytes with 6.5 nM [³H]24R,25(OH)₂D₃ for 10 s followed by subcellular fractionation. Cells were isolated by citrate chelation; everted duodenal loops (two loops in 60 ml of solution A) were stirred at room temperature for 15 min, the dissociated cells collected into 50 ml centrifuge tubes, and the duodenal loops incubated for an additional 15 min. The combined cells were collected by centrifugation (500 g, 5 min), the supernatant decanted, and the inside of the tube swabbed with a laboratory wipe while in the inverted position. The pellet was gently dispersed with a Teflon rod, and GBSS-BSA added drop-wise with stirring. Cells were resuspended in 20 ml, and then rapidly combined with an additional 20 ml GBSS-BSA containing 13 nM [³H]24R,25(OH)₂D₃, to give a final concentration of 6.5 nM steroid. The cells were swirled for 10 s, and then centrifuged (4 °C). The pellets were homogenized and fractionated as described in the legend to Fig. 1. Values represent means ± ranges for two independent experiments.

which upon binding of ligand, became internalized and sequestered in lysosomes. To assess such a possibility, authentic human DBP, lysosomal proteins and basal lateral proteins were separated on SDS-polyacrylamide gels for either Coomassie Blue staining, or for Western analyses. The composite shown in Fig. 3 demonstrates adequate protein staining of 1.5 µg of DBP (giving a single band), 100 µg of lysosomal protein, and 80 µg of basal lateral membrane protein. Western analyses of equivalent levels of DBP resulted in labeling of several bands (Fig. 3), one corresponding to the DBP protein band, while the smaller bands may have been due to nonspecific binding of secondary antibody to glycosylated contaminants. Lower levels (15–60 µg) of lysosomal or basal lateral proteins failed to give reaction product (data not shown), nor did 80–100 µg of protein (Fig. 3) at the molecular mass corresponding to DBP. Since the specificity of the commercially purchased antibody is not known, reaction product at lower molecular masses could be recognition of degradation products or cross-reactivity with related proteins/glycoproteins.

Characterization of the lysosomal binding activity for [3H]24R,25(OH)₂D₃

To assess certain properties of the lysosomal protein, time course studies and protein dependence of binding were

Figure 3 Western analyses of lysosomal (Ly) and basal lateral membrane (BLM) proteins with anti-DBP. The organelles of each fraction were combined within a group, and Percoll removed by dilution with greater than 10 vols of homogenization medium and centrifugation in a 45 Ti rotor (25 000 r.p.m., 1 h). Proteins (0.5, 100 and 80 µg of DBP, lysosomes and basal lateral membranes respectively) were separated on 8% SDS-polyacrylamide gels and either stained with Coomassie Brilliant Blue (left lanes), or blotted onto PVDF membranes for analyses with anti-DBP antisera, followed by alkaline phosphatase-conjugated secondary antibody (right lanes).

undertaken. Incubation of 50 µg of lysosomal protein with 16 nM [³H]24R,25(OH)₂D₃, in the absence or presence of excess unlabeled steroid (0–4 °C), produced an increase in specific binding between 0.5 and 2 h (data not shown). Increasing the duration of incubation did not result in further specific binding (data not shown). Using the 2-h incubation time, three levels of protein, 50, 100 and 155 µg in 0.2 ml final volume, were tested for specific binding. A linear increase ($P < 0.05$; $f(x) = -0.2303 \pm 137.5x$; $R^2 = 0.96$; $\text{adj}R^2 = 0.94$; $F = 49.73$; d.f. = 3) in specific [³H]24R,25(OH)₂D₃ binding was observed between 50 and 155 µg of protein.

Saturation analyses were conducted with both basal lateral membranes (Fig. 4A) and lysosomal fractions (Fig. 4B–D). Using 50 µg of protein per tube, samples were incubated with increasing concentrations of [³H]24R,25(OH)₂D₃ in the absence or presence of a 200-fold molar excess of unlabeled seco-steroid. Under these conditions, binding parameters for basal lateral

Figure 4 Saturation analyses of specific [^3H]24R,25(OH)₂D₃ binding to basal lateral membrane and lysosomal fractions. Isolated membranes or lysosomes were removed from Percoll as described in the legend to Fig. 3. Each concentration of steroid was tested (50 μg of protein per tube; 2 h, 0 °C) for total binding in triplicate with the indicated amount of labeled steroid, and in duplicate for nonspecific binding in the presence of a 200-fold molar excess of unlabeled steroid. Bound and free steroid were separated by HAP. Values represent means $\pm$ S.E.M.s for three independent experiments. (A) Saturation binding to basal lateral membranes; (B) saturation binding to lysosomal fractions; (C) Scatchard analyses of data from (B); (D) Hill analyses of data from (B). S_b , specifically bound hormone; S_f , free hormone.

Figure 5 Competition of various metabolites with [³H]24R,25(OH)₂D₃ for binding to lysosomal proteins. Lysosomal fractions were removed from Percoll as described in the legend to Fig. 3. All incubations were conducted in triplicate using 50 µg protein and 16 nM [³H]24R,25(OH)₂D₃ in the absence (total binding) or presence of the indicated unlabeled competitor. Specific binding for 24R,25(OH)₂D₃ was set to 100%. Results represent the means ± S.E.M.s for eight independent experiments. Letters different from each other represent significant differences ($P < 0.05$).

membranes gave an estimated $K_d = 8.5$ nM and $B_{max} = 149$ fmol/mg protein ($P < 0.05$; $R^2 = 0.49$; $\text{adj}R^2 = 0.41$; $F = 6.259$; d.f. = 15), and for lysosomal proteins $K_d = 7.4$ nM and $B_{max} = 142$ fmol/mg protein ($P < 0.05$; $R^2 = 0.58$; $\text{adj}R^2 = 0.53$; $F = 11.538$; d.f. = 19). Scatchard analyses of the binding data for lysosomes (Fig. 4C) yielded a non-linear curve, suggestive of cooperativity. Transformation of the data for Hill analyses (Fig. 4D) revealed a Hill coefficient (n_{app}) of 0.57 ± 0.11 ($P < 0.05$; $R^2 = 0.6$; $\text{adj}R^2 = 0.58$; $F = 27.196$; d.f. = 18), indicative of negative cooperativity. Scatchard and Hill analyses for [³H]24R,25(OH)₂D₃ binding to basal lateral membranes from chick intestinal epithelium have been presented elsewhere (Larsson *et al.* 1999, 2001).

To determine the specificity of binding, lysosomal proteins were incubated with [³H]24R,25(OH)₂D₃ in the absence or presence of various unlabeled competing metabolites (Fig. 5). When competition with 24R,25(OH)₂D₃ was set to 100%, the 24S,25(OH)₂D₃ isomer was found to give $81 \pm 7\%$ competition for binding ($n = 8$; $P > 0.05$, relative to competition with 24R,25(OH)₂D₃), while 25(OH)D₃ and 1,25(OH)₂D₃ produced $53 \pm 13\%$ and $39 \pm 11\%$ competition respectively ($n = 8$; each $P < 0.05$, relative to competition with 24R,25(OH)₂D₃).

Perfusion studies

The ability of 24S,25(OH)₂D₃ to compete efficiently with [³H]24R,25(OH)₂D₃ for binding to a lysosomal protein

Figure 6 Calcium transport in isolated duodenal loops perfused with control medium, 1,25(OH)₂D₃ or 1,25(OH)₂D₃ plus 24S,25(OH)₂D₃. Duodenal loops (three per group) of anesthetized chicks were cannulated through the celiac artery and perfused with control medium. The duodenal loop was removed, the celiac vein cannulated for collection of effluent, and the duodenal lumen cannulated for perfusion with ⁴⁵CaCl₂ in GBSS. After a 20-min basal period, and collection of the venous effluent during the latter 10 min, duodena were either continuously perfused through the artery with control medium for an additional 40-min treated phase, or with 130 pM 1,25(OH)₂D₃, or with 130 pM 1,25(OH)₂D₃ plus 6.5 nM 24S,25(OH)₂D₃. Samples were collected at 2-min intervals and aliquots removed for liquid scintillation spectrophotometry. Counts per min for each time point of the treated phase (T) were normalized to the average basal transport (av Basal) in corresponding preparations. Values represent means ± S.E.M.

was further tested in terms of physiological significance in the perfused duodenal loop system. Figure 6 depicts the results of duodena that were either vascularly perfused with control medium, 130 pM 1,25(OH)₂D₃ or 130 pM 1,25(OH)₂D₃ plus 6.5 nM 24S,25(OH)₂D₃. While 1,25(OH)₂D₃ stimulated calcium transport to levels that were 2.5-fold above controls, inclusion of 24S,25(OH)₂D₃ in the vascular perfusate abolished the stimulatory effect of 1,25(OH)₂D₃. These results implied a receptor-like function for the 24,25(OH)₂D₃ binding protein.

Protein purification

Optimal conditions for the extraction of the binding protein from lysosomal fractions were assessed by comparing homogenization with media containing detergent (10 mM CHAPSO, final concentration) and hypotonic media (TED buffer), followed by centrifugation at 160 000 g, 1 h. Since equivalent specific binding activity was extracted by either method, detergent was subsequently omitted. Elution of the crude lysosomal extract from an anion exchange column yielded the chromatogram shown in Fig. 7A. The fractions highest in specific [³H]24R,25(OH)₂D₃ binding (21–33) were pooled and concentrated. Analyses of content by SDS-PAGE and silver staining revealed numerous bands, necessitating a

further purification step on gel exclusion chromatography (Fig. 7B). Binding activity was concentrated in fractions 28–30, corresponding to a molecular mass of approximately 60 000. A representative silver-stained gel

is shown in Fig. 7C. The protein consistently present in active fractions corresponded to the 66 kDa band.

Discussion

Analyses of enterocyte subcellular organelles indicates that a specific binding protein for 24,25(OH)₂D₃ is enriched in lysosomal fractions, when binding is determined by the HAP method for separation of bound and free steroid. The lysosomal enrichment exceeds that of binding in basal lateral membrane fractions (Nemere *et al.* 1994, Fig. 1 of the current work), although affinity and sites/mg protein are similar (Fig. 4A and B). Isolated intestinal cells incubated with [³H]24R,25(OH)₂D₃ also sequestered the highest levels of seco-steroid in lysosomal fractions. Thus, both the unliganded and liganded forms of the 24R,25(OH)₂D₃ binding protein (24,25-BP) are localized in the same vesicles that are strong candidates as transporters of calcium and phosphate across the intestinal cell (Nemere *et al.* 1986, Nemere 1996b). Indeed, in mucosae from duodenal loops fractionated after vascular perfusion with 1,25(OH)₂D₃ and 24R,25(OH)₂D₃, lysosomal phosphate increases from the luminal media are blocked relative to equivalent preparations from tissue vascularly exposed to 1,25(OH)₂D₃ alone (Nemere 1996b). Thus, two possible explanations for the inhibitory actions of 24R,25(OH)₂D₃ on ion transport are (i) a direct effect of the liganded 24,25-BP on the lysosomal ATPase identified as the enzyme for loading the organelles with phosphate (Pisoni & Lindley 1992), and/or (ii) blocking of signal transduction pathways stimulated by 1,25(OH)₂D₃ (Nemere 1999).

A number of considerations indicate that the putative membrane receptor for 1,25(OH)₂D₃ and the 24,25-BP are different molecular entities. Neither protein exhibits substantial levels of competition for binding with heterologous ligand (Nemere *et al.* 1994, Fig. 5 in the current work). While the putative receptor for 1,25(OH)₂D₃ is

Figure 7 Isolation of a candidate 24,25-BP from lysosomal fractions. Fractions 1–3 from Percoll gradients were homogenized in 10–20 vols of TED (25 strokes on ice), centrifuged at 105 000 *g*, 1 h, and the supernatant passed through a syringe filter (0.45 µm) prior to concentration in spin columns. Concentrated supernatants were treated with protease inhibitors and frozen until further use. On the day of chromatography, aliquots were again filtered prior to application to an anion exchange column with a 1-ml injection loop. The column was washed for 7 min (1 ml/min flow rate), and then eluted with a linear 0–1 M NaCl gradient in 20 mM Tris, 1 mM EDTA, pH 8.0. (A) Representative chromatogram for anion exchange chromatography. Each fraction was assayed for specific [³H]24R,25(OH)₂D₃ binding and those with the highest activity were again concentrated and further fractionated on a sizing column. (B) Representative chromatogram for gel filtration. Each fraction was again assayed for specific [³H]24R,25(OH)₂D₃ binding and aliquots of active fractions run on SDS-PAGE with silver staining (C).

located largely in the basal lateral/endoplasmic reticulum (Golgi) fractions (Nemere *et al.* 1994, 2000), the 24,25-BP is enriched in lysosomes when equivalent subcellular fractionation protocols are used. Electron microscopy of thin sections labeled with Ab099 directed against the putative membrane receptor for 1,25(OH)₂D₃ indicates that exposure of intestinal tissue to 1,25(OH)₂D₃, but not 24R,25(OH)₂D₃, results in apparent translocation of the antigen to the nucleus. In contrast, both liganded and unliganded forms of the 24,25-BP appear to be in lysosomes (Figs 1 and 2). A definitive relationship or lack of homology between the two proteins will be provided by N-terminal microsequencing of the 24,25-BP for comparison with the sequence obtained from the putative 1,25(OH)₂D₃ membrane receptor.

The enrichment of the 24,25-BP in lysosomal fractions suggested that the protein might be the serum transporter DBP, which through endocytosis, became sequestered in vesicles. However, Western analyses with antibody against authentic DBP failed to detect a reaction product in either lysosomal or basal lateral membrane fractions. The possibility remains that the 24,25-BP may have structural homology to the intracellular DBP characterized in New World primate cells (Gacad *et al.* 1997, Wu *et al.* 2000), although competition studies indicate a greater affinity for 24R,25(OH)₂D₃ over 25(OH)₂D₃. Work is in progress to obtain the sequence of the 24,25-BP which will provide definitive data regarding potential similarities to the 25(OH)₂D₃ binding protein; the latter protein has been shown to be related to heat shock proteins (Gacad *et al.* 1997, Wu *et al.* 2000).

Competition studies also indicate that the 24S,25(OH)₂D₃ isomer has a high affinity for the lysosomal 24,25-BP. Since both isomers exist naturally (Ishizuka *et al.* 1984), this result is not unexpected. Nor is the finding that 24S,25(OH)₂D₃ is effective in inhibiting 1,25(OH)₂D₃-stimulated calcium transport in the perfused duodenal system, since the physiological activity agrees with the competitive binding data.

The binding of [³H]24R,25(OH)₂D₃ to lysosomal and basolateral membranes showed no difference in affinity or number of receptors/mg protein. This suggests that the 24R,25(OH)₂D₃ binding proteins in lysosomal and basal lateral membranes are of a common origin. However, one difference between the lysosomal and basal lateral binding proteins is in analyses of cooperativity. In basal lateral membranes, binding exhibits positive cooperativity (Larsson *et al.* 1999, 2001), while in lysosomal fractions binding exhibits negative cooperativity. One explanation for these observations might be that the binding proteins in the two subcellular compartments have different naturally occurring ligands, resulting in differences in cooperative binding. Efforts are currently underway to purify sufficient quantities of these two 24,25-BP for sequencing, which in turn may yield additional information about the observed differences in cooperativity.

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