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Evidence That Annexin II Is not a Putative Membrane Receptor for 1α,25(OH)$_2$-Vitamin D$_3$

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Abstract The seco-steroid hormone 1α,25(OH)$_2$-vitamin D$_3$ (1,25-D$_3$) is known to generate biological responses via both genomic and non-genomic rapid signal transduction pathways. The calcium regulated annexin II/p11 heterotetramer (AII$_2$/p11$_2$] was proposed by Baran and co-authors to be the membrane receptor responsible for mediating non-genomic, rapid actions of 1,25-D$_3$, based on ligand affinity labeling, competition, and saturation analysis experiments. Given the cytosolic presence of both the monomeric and heterotetrameric form of AII and their functional regulation by intracellular calcium concentrations, which are known to be affected by 1,25-D$_3$ rapid, non-genomic activities, we investigated in vitro the affinity of [^{3}H]1,25-D$_3$ for the AII monomer and AII$_2$/p11$_2$ in the absence and presence of calcium using saturation analysis and gel-filtration chromatography. Using two different techniques for separating bound from free ligand (perchlorate and hydroxylapatite (HAP)) over a series of 30 experiments, no evidence for specific binding of [^{3}H]1,25-D$_3$ was obtained with or without the presence of 700 nM exogenous calcium, using either the AII monomer or AII$_2$/p11$_2$. However saturable binding of [^{3}H]1,25-D$_3$ to the lipid raft/caveolae enriched rat intestinal fraction was consistently observed ($K_d = 3.0$ nM; $B_{max} = 45$ fmols/mg total protein). AII was detected in lipid raft/caveolae enriched fractions from rat and mouse intestine and ROS 17/2.8 and NB4 cells by Western blot, but incubation in the presence of exogenous calcium did not ablate 1,25-D$_3$ binding as reported by Baran et al. Our results suggest that AII does not bind 1,25-D$_3$ in a physiologically relevant manner; however, recent studies linking AII$_2$/p11$_2$ phosphorylation to vesicle fusion and its calcium regulated localization may make AII a possible down-stream substrate for 1,25-D$_3$ induced rapid cellular effects. J. Cell. Biochem. 91: 852–863, 2004.

Key words: vitamin D; 1α,25(OH)$_2$-vitamin D$_3$; annexin II; calcium; non-genomic responses; rapid responses

The seco-steroid 1α,25-dihydroxyvitamin D$_3$ (1,25-D$_3$) generates biological responses via both genomic and non-genomic, rapid activation of ion channels and signal transduction pathways [Bouillon et al., 1995; Norman et al., 2001b, 2002a]. 1,25-D$_3$, a biologically active metabolite of vitamin D$_3$, regulates gene transcription via interaction with a well-characterized nuclear vitamin D receptor (VDR_{nuc}), which interacts with hormone response elements (HREs) present in the promoter of the target genes [Norman and Collins, 2001a]. Responses initiated at or near the plasma membrane have been proposed to be mediated by a putative membrane receptor (VDR_{mem}) [Nemere et al., 1994; Norman et al., 1997a], which upon binding 1,25-D$_3$ and other specific analog conformers [Norman et al., 1997b] has been shown to activate the following responses: opening of voltage gated Ca^{2+} [Caffrey and Farach-Carson, 1989] and chloride channels in osteoblasts [Zanello and Norman, 1997]; upregulation of phosphatidylinositol 3′-kinase (PI3K) in vascular smooth muscle [Rebsamen et al., 2002]; stimulation of PKC in growth zone condrocytes via activation of phospholipase C and phospholipase A$_2$ (PLA$_2$) [Sylvia et al., 1998]; activation or inhibition of specific PKC isoforms [Schwartz et al., 2002] in plasma membranes and matrix vesicles isolated from costochondral growth zone cartilage cells; stimulation of intestinal Ca^{2+} transport in chick intestine, termed transcaltachia [Nemere et al., 1984], and secretion of insulin

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from isolated rat pancreatic islets [Kajikawa et al., 1999].

The first report and isolation of a possible protein/receptor for 1,25-D$_3$ present in the basal lateral membrane of chick intestinal cells mediating the rapid, non-genomic transport of calcium (transcaltachia) described difficulty in the quantification and purification of the protein necessary for its characterization [Nemere et al., 1994]. An antibody (Ab99) later generated against this protein has been shown to block various rapid responses elicited by 1,25-D$_3$ suggesting this protein may function as a VDR$_{mem}$ [Nemere et al., 1998]. These findings have led to the proposal that separate membrane receptors exist for 1,25-D$_3$ and its 1-deoxy metabolite 24R,25(OH)$_2$-D$_3$ [Pedrozo et al., 1999; Boyan et al., 2002]. In separate experiments, Baran et al. [2000a,b] reported that they had successfully isolated the VDR$_{mem}$ via in vitro labeling of AII with a [^{14}C]-labeled bromoacetate analog of 1,25-D$_3$ (Fig. 1) designed originally to form a covalent adduct with putative H-bonding partners present in the VDR$_{nuc}$ ligand binding domain (LBD) [Ray et al., 1996]. Also several groups have proposed that the VDR$_{nuc}$ may function as a VDR$_{mem}$ [Barsony et al., 1995; Bhatia et al., 1995; Kim et al., 1996; Norman et al., 2002b], but further experimentation is needed to solidify this proposal. Another recent report provides evidence that 1,25-D$_3$ binds to the lipid raft/caveolae enriched membrane fraction (CMF) isolated from chick duodenal mucosa with specificity and high affinity [Norman et al., 2002b].

The annexins are a family of 13 proteins that characteristically bind to cellular membranes

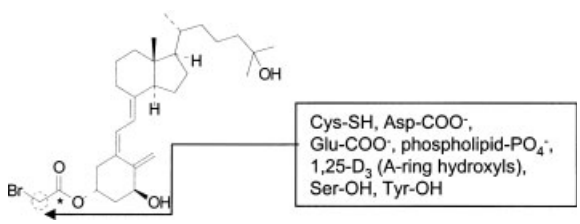

Fig. 1. Reactivity and affinity labeling of annexin II (AII). Structure of the 1α,25(OH)$_2$-vitamin D$_3$ (1,25-D$_3$)[^{14}C]-bromoactetate [Ray et al., 1996] used by Baran et al. in the affinity labeling of partially purified plasma membranes and pure AII/p11 heterotetramer. The alkylation/cross-linking site is circled and the [^{14}C] carbon labeled (*). Amino acids and other possible nucleophiles that could possibly react with the bromoacetate analog under Baran's experimental conditions are listed in the box.

through a Ca^{2+}-dependent interaction with anionic phospholipids enriched in the cytosolic leaflets (lipid rafts), a unique property that distinguishes the annexins from most other calcium binding proteins; see Waisman [1995] for review. The annexin Ca^{2+} binding sites are composed of two anionic charged residues and a hydroxyl group, not a typical EF2 hand motif [Waisman, 1995]. By definition an annexin must also contain a conserved structural element, the so-called annexin repeat, a segment of some 70 amino acids (see Fig. 2); for detailed structural information see Burger et al. [1996]. Recently several members of the annexin family have been identified in lipid raft preparations, with their presence there, in most cases, regulated by Ca^{2+} [Gerke and Moss, 1997, 2002]. This specific association of annexin with the cytoplasmic side of rafts has led to the hypothesis that annexins play a role in regulating their assembly and dynamics [Harder et al., 1997; Oliferenko et al., 1999; Babiychuk and Draeger, 2000; Corvera et al., 2000; Prevostel et al., 2000].

Annexin II (AII) is unique amongst the annexin family because it forms a heterotetramer (AII$_2$/p11$_2$) by association of two AII monomers, which each binds a small S100 peptide subunit, p11, at resting intracellular Ca^{2+} concentrations (Fig. 2) [Gerke and Moss, 1997]. This property allows AII$_2$/p11$_2$ to associate with lipid rafts and F-actin via the common annexin–calcium–phospholipid bridge (Ca^{2+}-dependent) [Waisman, 1995; Filipenko and Waisman, 2001] and to chelate with raft structures rich in cholesterol [Corvera et al., 2000; Zeuschner et al., 2001; Harder et al., 1997]. Other unique properties that distinguish AII from other members of the family are its ability to mediate intracellular vesicle aggregation and fusion [Kirsch et al., 2000], its participation in cell–cell adhesion [Hansen et al., 2002], and its secretion in keratinocytes [Karimi-Busheri et al., 2002] and in response to insulin receptor activation [Zhao et al., 2002].

The AII monomer and AII$_2$/p11$_2$ have unique functions in vivo. The monomer has been shown to function in a calcium-independent manner interacting with cholesterol-rich endosomal membranes via its NH_2-terminus [Jost et al., 1997; Konig and Gerke, 2000]. However, the binding of the heterotetramer to most lipid membranes is calcium-dependent and mediated by the annexin core repeat (Fig. 2)

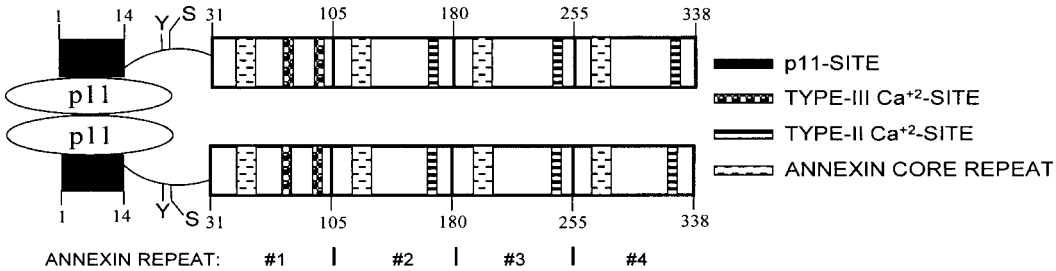

Fig. 2. Structural organization of AII heterotetramer. AII is a protein of 338 aa. The four annexin repeats (~70–80 amino acids) [Barton et al., 1991] are shown as large rectangles with amino acid numbers separating each repeat. Each repeat contains the highly conserved endonexin fold, annexin core-repeat, and is schematically represented by rectangles filled with solid diamonds. The first repeat contains two type-III Ca^{2+} binding sites while each of repeats 2,3,4 contain a single type-II Ca^{2+} binding site [Connolly et al., 1991; Jost et al., 1994]. Residues 1–14 of the AII N-terminus, shown in black, have a very high affinity ~30 nM for the S100 protein family member, p11 (11 KDa). This complex is formed spontaneously at resting calcium intracellular concentrations. The function of AII is regulated by phosphorylation of three different residues (S11, Y23, and S25) and subsequent phosphatase and protease activity [Babiychuk et al., 2002] Taken together AII functioning is believed to be highly regulated by the intracellular presence of p11, by intracellular calcium concentrations, and by activated cytoplasmic signaling pathways related to the physiological state of the cell (for review see Gerke and Moss [2002]).

[Ayala-Sanmartin et al., 2000]. The N-terminus of AII is a substrate for kinases [Rothhut, 1997] and other regulatory modifications [Gerke and Moss, 2002] which coupled with its calcium-independent and -dependent cytosolic activities, makes it an attractive nominee for participating in rapid, non-genomic activity induced by 1,25-D_3.

Given the calcium-independent and -dependent functioning of AII and the recent evidence suggesting that the heterotetramer forms a covalent adduct with the [^{14}C]-bromoacetate analog of 1,25-D_3 (Fig. 1), we investigated whether 1,25-D_3 binding occurred before or after the formation of the AII/p11 heterotetramer. In doing so, it was found that neither the AII monomer nor the heterotetramer had any affinity for 1,25-D_3. However saturable binding of [^{3}H]-1,25D_3 to a lipid raft/CMF from rat intestine was observed. Collectively this is evidence that AII may not be a VDR_{mem} receptor.

METHODS

Reagents

1α,25-dihydroxy[23,24(n)-^{3}H]cholecalciferol ([^{3}H]1α,25$(OH)_2D_3$) and 25-hydroxy[26,27-^{3}H]-cholecalciferol ([^{3}H]25$(OH)D_3$) were obtained from Amersham Pharmacia (Piscataway, NJ). 1α,25$(OH)_2D_3$ was kindly provided by Dr. M.R. Uskokovic (Hoffmann-La Roche, Nutley, NJ). The AII/p11 heterotetramer from cow lung was purchased from Biodesign International (Saco, ME). The AII A66E mutant construct, composed of the pSE420 expression vector and the human *AII* gene sequence, was a kind gift from Prof. Hartmut Luecke of the Department of Molecular Biology and Biochemistry, University of California, Irvine. The annexin mutation was introduced by Dr. Volke Gerke of the Westfälische Wilhelms Universität in Germany. This mutation, made for crystallization purposes, does not affect the functioning of AII (personal communication from Dr. H. Luecke). Vitamin D binding protein (DBP) was a gift from Profs. Roger Boullion and Hugo Van Baelen (LEGENDO, Katholieke Universiteit, Leuven, Belgium).

Saturation Analysis

Ligand saturation analysis of AII or other binding proteins was carried out over a range of 0.25–30 nM [^{3}H]1α,25$(OH)_2D_3$ (88.0 Ci/mmol; 195,000 dpm/pmole) in the absence (three replicates) and presence (two replicates) of 100-fold excess cold 1α,25$(OH)_2D_3$. Borosilicate glass tubes containing the protein/ligand mixture in TED buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 1 mg/ml gelatin, pH = 7.4) were placed on ice and incubated for 12 h. The total volume in each tube was always 273 μl, of which 8.5% was organic (EtOH). Incubation buffer with 700 nM $CaCl_2$ contained all contents of the TED buffer, but was devoid of EDTA. After the incubation was complete, the protein-bound [^{3}H]1α,25$(OH)_2D_3$ was separated from free [^{3}H]1α,25$(OH)_2D_3$ following the

perchlorate method used by Baran et al. [2000b] or our standard hydroxylapatite (HAP) assay [Wecksler and Norman, 1980]. The perchlorate method is an acid precipitation, whereby perchlorate addition causes precipitation of the VDR, separating the protein-bound and free ligand. HAP binds the VDR thus separating the protein-bound from free ligand. The major difference between the two assays is that the HAP pellet is washed 3× with 0.5% Triton ×100 and the perchlorate pellet is not. Incubations were conducted using a large excess of the AII heterotetramer ($AII_2/p11_2$) or the AII monomer (A66E) with respect to the ligand concentration.

Sephacryl S-200 Gel-Filtration

Sephacryl S-200 High Resolution resin (Amersham Pharmacia) was loaded and seeded following factory protocol in a 16 mm/60 cm gel-filtration column (Amersham Pharmacia), followed by equilibration with Ca^{2+} free (TED devoid of gelatin) or TED buffer containing 700 nM $CaCl_2$ devoid of gelatin. The column was calibrated using factory recommended standards. AII monomer (1.5 mg) was incubated with 1.5 molar equivalents of [^{3}H]1α,25(OH)$_2$D$_3$ (88.0 Ci/mmol; 10 dpm/pmole) following the protocol described in "Saturation Analysis," with the exception that after the 12 h incubation the sample was loaded onto the column. The column was then run at a flow rate of 1.3 ml/6 min and 0.5 ml fractions collected. The fractions of interest were lyophilized to dryness and resuspended in 100 μl of ddH_2O and OD_{280} ($\varepsilon = 30{,}250\ M^{-1}cm^{-1}$) taken to assess protein concentration. The cell was then washed 3× with 10% EtOH/H_2O and contents transferred to a scintillation vial containing 10 ml of Liquiscint scintillation cocktail (National Diagnostics, Manville, NJ). The sample vials were then placed in an LS6500 liquid scintillation spectrometer (Beckman Inst., Fullerton, CA) to measure the amount of [^{3}H]1,25(OH)$_2$D$_3$ present in each fraction of interest. The total amount of protein and ligand eluted was then calculated and compared to the amounts added in the original incubation mixture. The control experiment where the vitamin D binding protein (DBP; $\varepsilon = 28{,}840\ M^{-1}\ cm^{-1}$) was incubated with 1.12 equivalents [^{3}H]25(OH)-D$_3$ was carried out in the same manner described above, with the exception that 20 mM K_2HPO_4 (pH = 8.6) was used as the buffer.

Electrophoresis and Immunoblots

Lipid raft/CMF, isolated as previously described [Norman et al., 2002b] and controls were subjected to SDS/PAGE using 10–20% Tris–glycine PAGE Gold precast gels (BioWhittaker Molecular Applications; Rockland, ME) followed by semi-dry electrotransfer to Immobilon-P PVDF membranes (Millipore; Bedford, MA). PVDF membranes were incubated in blocking buffer (5% non-fat dried milk (w/v) in Tris-buffered saline—0.05% Tween [TBST]) and subsequently incubated with mouse anti-AII antibody (IgG_1). Primary antibody incubations were for 3 h at a 0.5 μg/ml concentration. After washing five-times in TBST, the membranes were incubated in a 1/1,000 dilution of goat anti-mouse IgG-HRP (Bio-Rad; Hercules, CA) for 1 h. After washing seven-times in TBST, the membranes were developed by enhanced chemiluminescence using SuperSignal West Pico Kit substrate reagents (Pierce; Rockford, IL). Protein concentrations were determined by the Bradford method according to the instructions supplied by the manufacturer of the dye (Bio-Rad) using bovine serum albumin (1 mg/ml) as the standard (Sigma, St. Louis, MO). The CMF loading concentrations are given in the figure legend.

RESULTS

In a series of experiments designed to replicate the saturation analysis performed by Baran et al., pure AII/p11 heterotetramer ($AII_2/p11_2$) (2.2 μg/tube; ~200 nM) was incubated with up to 4 nM [^{3}H]1,25-D$_3$ and the bound and free ligand separated using the perchlorate method [Nemere et al., 1994]. In five independent binding experiments using a 50-fold excess of $AII_2/p11_2$, only a small degree of random binding was observed (Fig. 3A,B). Such an excess of AII in the incubation experiments would never be expected to produce saturable binding; however, if the affinity for the ligand is high, a condition expected for formation of a physiological complex that elicits a rapid cellular event, then the specific binding should produce a linear line whose values are approximately equal to the total binding when purified protein samples are used.

Given Baran's reported K_d of the pure $AII_2/p11_2$ (2.4 and 5.5 nM) [Baran et al., 2000a,b] for [^{3}H]1,25-D$_3$, further experiments were carried out with increased concentrations of [^{3}H]1,25-

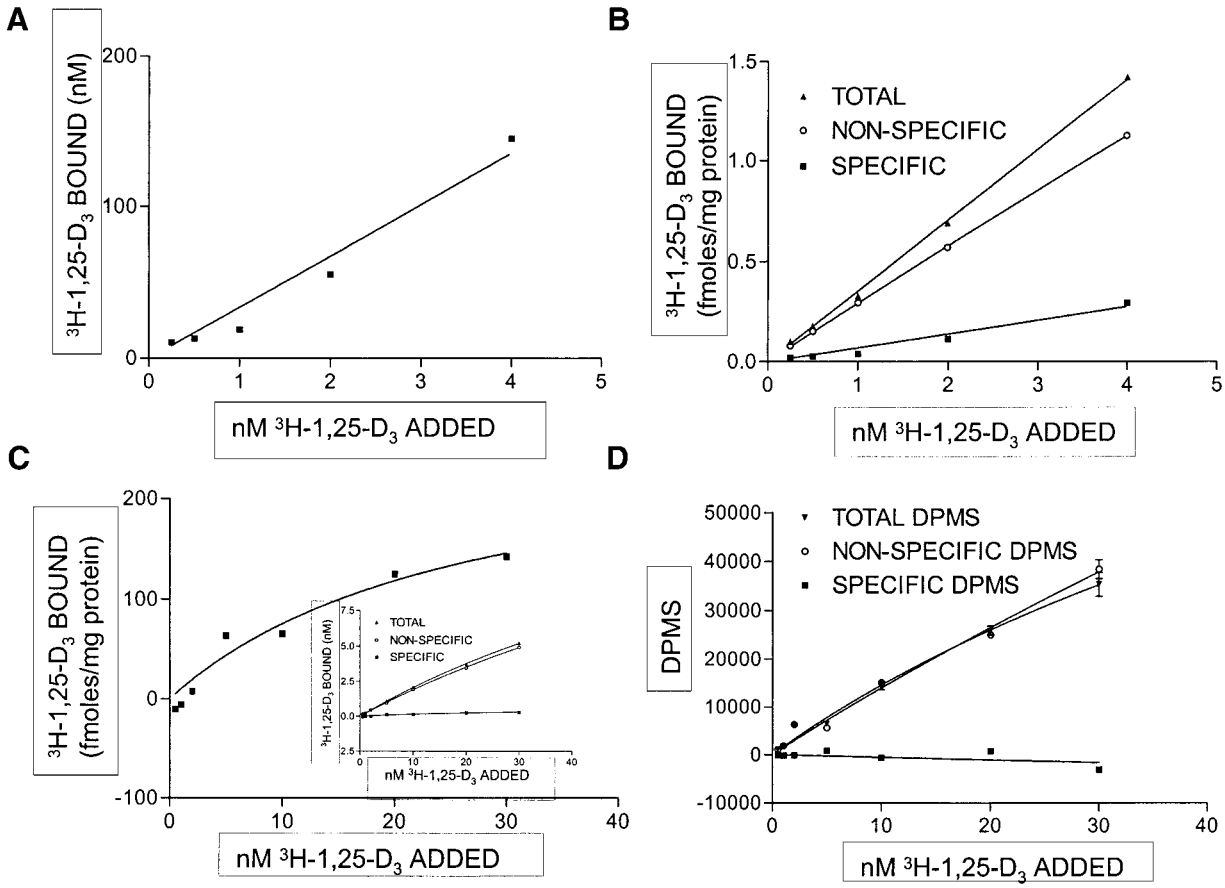

Fig. 3. 1α,25(OH)$_2$D$_3$ saturation analysis of AII/pII heterotetramer (2.2 μg/tube; 2.0×10^{-7} M) using the perchlorate method for separation of bound from free radioactive ligands. **A/B**: Presents results from an exact replicate of the saturation experiment performed by Baran et al. [2000b], with the additional concentration of 0.25 nM [^{3}H]1α,25(OH)$_2$D$_3$ added. Two plots are depicted; (A) where [^{3}H]1α,25(OH)$_2$D$_3$ bound (total, non-specific, and specific) is expressed in nM and (B) as fmol/μg protein. **C**: Transformed data from the "best" experiment (n = 2), where [^{3}H]1α,25(OH)$_2$D$_3$ is expressed as fmol/μg protein; "best" implies some evidence of saturable 1,25-D$_3$ binding. Non-linear regression of this curve produced an r^2-value of 0.95, while linear regression gave an $r^2 = 0.89$. The inset panel shows the amount of total, non-specific and specific binding (nM bound) at the given concentrations of [^{3}H]1α,25(OH)$_2$D$_3$ added. The calculated K_d was 25.7 nM while the $B_{max} = 1230$ fmol/μg protein. **D**: Non-transformed experimental data from a typical experiment (n = 11) where [^{3}H]1α,25(OH)$_2$D$_3$ binding (total, non-specific, and specific) is expressed as DPM.

D$_3$ in an attempt to investigate if the low specific binding was due to a low degree of functional protein in the sample tube under the incubation conditions. Of the 12 experiments performed incubating AII$_2$/p11$_2$ with up to 30 nM [^{3}H]1,25-D$_3$, only 2 produced plausible saturation curves with only a very small degree of specific binding and with the majority of the total [^{3}H]1,25-D$_3$ being present in the non-specific fraction (Fig. 3C). These curves gave K_d-values approximately 50–100-fold higher than Baran's reported values (10.3 and 2.5 nM) for the partially purified membrane fraction from ROS 24/1 cells and pure AII$_2$/p11$_2$, respectively. The amount of bound radioactivity observed in these experiments (Fig. 3D) definitely falls within the range needed for high precision and accuracy in the experiment; however, the calculated specific binding in each case seemed random, sometimes even negative.

We then modified the binding assay by removing CHAPSO detergent from the incubation buffer and changing the technique used to separate the bound and free metabolites to HAP, due to its use in the purification of AII$_2$/p11$_2$ from bovine lung (Biodesign International) and the low amount of non-specific binding observed in the presence of a protein with high affinity for 1,25-D$_3$ [Wecksler and Norman, 1980]. These results (Fig. 4) show very low amount of specific binding of AII$_2$/p11$_2$ (22.5 nM) through the range of 2.5–25 nM

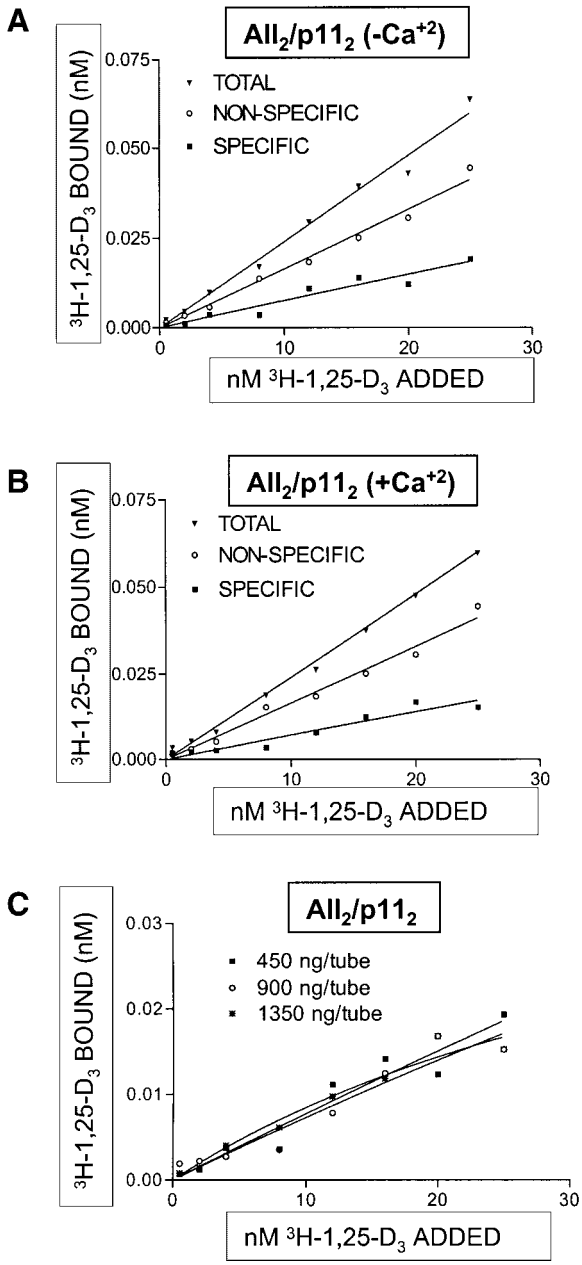

Fig. 4. 1α,25(OH)$_2$D$_3$ saturation analysis of AII/pII heterotetramer and rat intestinal CMF using the hydroxyapatite (HAP) method to separate bound from free ligand. **A**: AII/p11, 0.45 μg/tube (2.25×10^{-8} M), was incubated with the given amounts of [^{3}H]1α,25(OH)$_2$D$_3$ in the absence of Ca^{2+} in the buffer. Experimental data including total, non-specific, and specific binding observed after the 4 h incubation. Both linear and non-linear regression of the specific binding produced r^2 values of 0.93, validating the randomness of the data. **B**: AII/p11, 0.45 μg/tube (2.25×10^{-8} M), was incubated with the given amounts of [^{3}H]1α,25(OH)$_2$D$_3$ in the presence of 700 nM Ca^{2+}. Linear and non-linear regression of the specific binding produced $r^2 = 0.92$. **C**: Three putative saturation curves obtained with varying concentrations [450 (22.5 nM), 900 (45 nM), and 1,350 μg/tube (67.5 nM)] of AII/p11 are shown.

[^{3}H]1,25-D$_3$ added (Fig. 4A). Experiments using subnanomolar and micromolar concentrations of AII were also performed and produced identical results (data not shown).

Even though the addition of calcium to the incubation buffer has been reported to inhibit AII$_2$/p11$_2$ binding of the [^{14}C]-bromoacetate analog of 1,25-D$_3$ [Baran et al., 2000a] no suppression of specific, non-specific, or total binding was observed when 700 nM Ca^{2+} was present in the incubation buffer (Fig. 4B). Increasing the concentration of AII$_2$/p11$_2$ in each tube from 22.5 to 67.5 nM produced no change in the amount of specific binding observed (Fig. 4C); furthermore, no change was observed in the non-specific and total dpm (data not shown). Taken together, even at very high concentrations of protein, AII$_2$/p11$_2$ did not bind [^{3}H]1,25-D$_3$ at physiological pH in the presence or absence of calcium.

Figure 5A is a representative saturation curve from incubation of rat lipid raft/CMF from duodenal mucsoa with 1,25-D$_3$ ($K_d = 3.0$ nM). AII is found in rat CMF (Fig. 7A) and, therefore, given the presence of membrane lipids and accessory proteins AII could have all the thermodynamic counterparts needed to achieve the optimal fold needed for 1,25-D$_3$ binding in this in vitro assay. To test whether this was a possibility we added exogenous calcium to the incubation mixtures, because it was shown by Baran et al. [2000b] that the degree of alkylation by the affinity label (Fig. 1) was reduced in the presence of 600 nM exogenous Ca^{2+}. Even adding mM Ca^{2+} concentrations to the incubation buffer produced no change in the amount of specific binding of [^{3}H]1,25-D$_3$ to the rat intestinal CMF (Fig. 5B).

Because the AII monomer has been shown to reside in the cytosol and have a unique function in the absence of calcium and p11, we investigated whether the results obtained by Baran et al. could be explained by the covalent modification of the monomer by the bromoacetate analog, prior to formation of the heterotetramer in vivo. A mutant form of the AII monomer (A66E), which displayed no altered activities (Dr. H. Luecke, personal communication) and cross-reacted with AII antibody (Fig. 7C) was used in Sephacryl S-200 gel-filtration (Fig. 5) and saturation analysis (data not shown) in the presence of [^{3}H]1,25-D$_3$ in order to evaluate the possibility that binding of 1,25-D$_3$ to AII was prior to heterotetramer

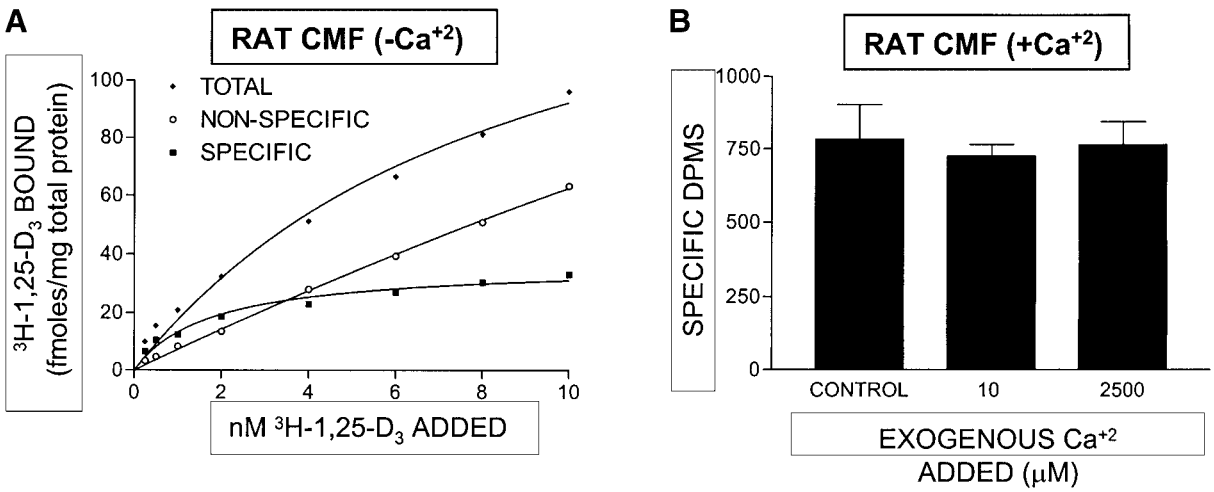

Fig. 5. **A**: Positive control for HAP protocol. Rat intestinal CMF (n = 3) was incubated with [^{3}H]1α,25(OH)$_2$D$_3$ (0.25–10 nM) producing a $K_d = 3.0$ nM (specific $r^2 = 0.98$). **B**: Incubation of rat intestinal CMF with 8 nM [^{3}H]1α,25(OH)$_2$D$_3$ in the absence (control) or presence of 10 μM and 2.5 mM exogenous Ca^{2+}.

formation. AII (A66E; 0.125 mM) was incubated with 1.5 equivalents of [^{3}H]1,25-D$_3$ in the absence (Fig. 6A) and presence (Fig. 6B) of 700 nM Ca^{2+}. Under neither condition was binding activity comparable to the control filtration experiment using the vitamin D binding protein, DBP, whose affinity for the vitamin D metabolite 25(OH)-D$_3$ is comparable to the proposed AII affinity for 1,25-D$_3$ (Fig. 6C). Only 400–500 pmoles of [^{3}H]1,25-D$_3$ was detected in the pooled fractions containing approximately 42,000 pmoles of AII.

If the activation of any physiological AII activities occurs in response to 1,25-D$_3$ it is important to evaluate AIIs presence in cell and tissue types known to specifically bind 1,25-D$_3$ at the cell membrane level. In order to probe for the presence of AII in CMF from rat and mouse intestinal mucosa, ROS 17/2.8 cells (rat), and NB4 cells (human), Western analysis using a mouse monoclonal COOH-terminal antibody to AII was performed (Fig. 7A,B). Visualization of AII from CMF fractions isolated from cultured ROS 17/2.8 and NB4 cells (Fig. 7B) seems to indicate a lower concentration of AII compared to intestinal tissue homogenates. This could both be indicative of decreased p11 subunit concentrations and, therefore, reflect the decrease of plasma membrane localized AII in the cell cultures or could be due to increased phosphorylation of the N-terminal of AII in ROS 17/2.8 and NB4 cells thereby decreasing the amount of detectable, unmodified AII.

DISCUSSION

The calcium regulated AII/p11 heterotetramer was proposed by Baran et al. [2000a,b] to be the membrane receptor for the steroid hormone, 1,25-D$_3$, based on affinity labeling, competition, and saturation analysis experiments. They performed four point saturation analysis on isoelectrically purified plasma membrane proteins obtained from ROS 24/1 cells and also on pure bovine AII$_2$/p11$_2$. They reported K_d values for [^{3}H]1,25-D$_3$ of 10.3 and 2.4 nM, respectively, for the ROS 24/1 cells and pure heterotetramer [Baran et al., 2000b]. In addition, no visible nonspecific binding was observed in these experiments (see their Fig. 3A,B) [Baran et al., 2000b]. That report also showed that pretreatment of the ROS 24/1 cells with anti-AII abolished the 1,25-D$_3$ induced increase in intracellular calcium; however, (see their Fig. 9B (control)) [Baran et al., 2000b] already shows a diffuse fluorescence signal for calcium in the cytoplasm prior to treatment with 1,25-D$_3$. A second report [Baran et al., 2000a] using the same ROS 24/1 cells again utilizing only a four point saturation analysis yielded K_d-values of 5.5 nM for [^{3}H]1α,25-D$_3$ and 6.0 nM for [^{3}H]1β,25-D$_3$. Calcium was also shown to inhibit, in a concentration dependent manner, the binding of the [^{14}C]-bromoacetate analog (Fig. 1) in that report.

A key aspect in our [^{3}H]1,25-D$_3$ saturation analysis of AII was to include many more ligand concentrations than were typically used in the

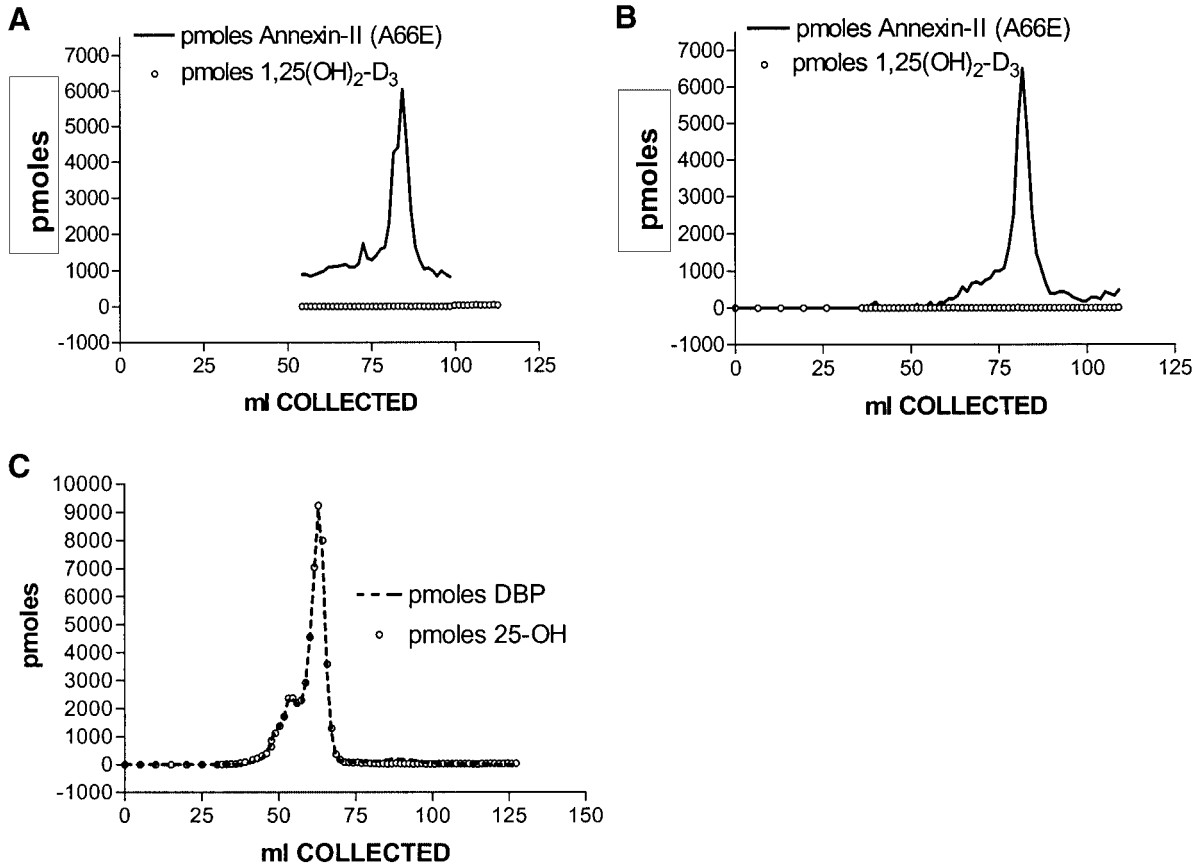

Fig. 6. Sephacryl S-200 gel-filtration chromatography of AII (A66E) monomer with [^{3}H]1α,25(OH)$_2$D$_3$. **A**: The chromatogram of 1.5 mg (0.125 mM) of AII incubated with 1.5 equivalents of [^{3}H]1α,25(OH)$_2$D$_3$ in the absence of Ca^{2+}. No co-elution of ligand with the protein was observed. **B**: The elution profile of 1.5 mg (0.125 mM) of AII in the presence of 700 nM Ca^{2+} is depicted, "no co-elution of [^{3}H]1α,25(OH)$_2$D$_3$ with the AII monomer was observed". **C**: A positive control where DBP, vitamin D binding protein (0.175 mM), was incubated with its wild type ligand 25(OH)-D$_3$ (1.35 equivalents). The K_d-value for DBP/25(OH)-D$_3$ complex is 3.0 nM [Haddad and Walgate, 1976] and, therefore, is equivalent to the reported K_d-value of 1α,25(OH)$_2$D$_3$ for AII/pII [Baran et al., 2000b].

Baran studies (e.g., 8 versus 4). The failure to achieve saturable or high amounts of specific binding, coupled with no observed co-elution of [^{3}H]1,25-D$_3$ in gel-filtration experiments, where near millimolar concentrations of highly purified AII were used in the incubation, lead us to conclude that AII does not specifically bind 1,25-D$_3$. Importantly our hydroxyl apatite procedure for separation of bound-from-free ligand was able to demonstrate specific binding of [^{3}H]1,25-D$_3$ to the lipid raft/CMF isolated from rat intestinal mucosa (Fig. 7A). This result serves as a positive control for our AII binding studies.

In this communication binding of [^{3}H]1,25-D$_3$ to rat intestine CMF is comparable to our earlier results reported for chick intestinal mucosa CMF [Norman et al., 2002b]. The presence of AII in the membrane enriched fraction (Fig. 7A) is expected and the observed 1,25-D$_3$ binding is unlikely due to the presence of AII, because the receptor(s) mediating rapid cellular events induced upon 1,25-D$_3$ binding would be expected to bind the ligand with high affinity and specificity [Sweet and Murdock, 1987] since the homeostatic circulating concentration of the hormone is ≈50 pg/ml (~150 pM).

Based on the in vitro results shown in Figures 3–5, neither the AII monomer nor the AII/p11 heterotetramer were found to bind 1,25-D$_3$ with high affinity or specificity, suggesting that it is not a putative membrane receptor responsible for the rapid actions mediated by the hormone. An argument could be made here that AII could bind 1,25-D$_3$ with intermediate affinity (10^{-7}–10^{-8} M) due to its increased local concentrations in structured microdomains, like caveolae; however, this is unlikely given

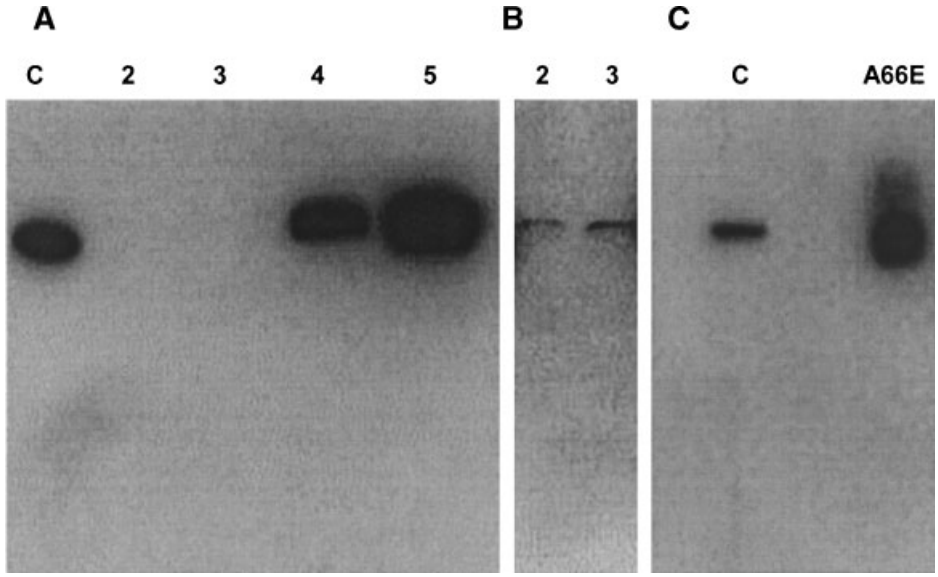

Fig. 7. Immunoblot analysis of AII in the caveolae enriched membrane fractions (CMFs). **A**: Immunoblot detection of AII. Loading concentrations were as follows: c, Zymed Laboratories, Inc., positive MDCK AII positive control 2 μl; **lane 2**, approximately 10 μg of NB4 CMF; **lane 3**, approximately 10 μg of ROS17/2.8 CMF; **lane 4**, 10 μg of rat intestinal CMF; **lane 5**, 2 μg of mouse intestinal CMF; A66E, 2 μl of AII monomer (A66E). **B**: Overexposure of lanes 2 and 3 of **panel A**. **C**: Immunoblot of AII monomer (A66E) and positive control Zymed Laboratories, Inc., positive MDCK AII (c). Control AII supplied with the antibody migrates at approximately 38 kDa.

that the binding of the [^{14}C]-bromoacetate of 1,25-D_3 was shown to be inhibited by addition of 600 nM exogenous Ca^{2+} [Baran et al., 2000a] a condition required for AII/p11 plasma membrane association. In addition nearly all experiments performed contained 25–200 nM AII, sufficient to display measurable binding of an intermediate affinity ligand.

In order to validate that a protein receptor binds a ligand in a specific and physiological relevant manner, one must demonstrate affinity, activity, and specificity under whole cell or in vivo circumstances. The results implying specific recognition and subsequent binding of 1,25-D_3 to AII presented by Baran et al. could possibly be explained by the chemical reactivity and specificity of the 1α,25$(OH)_2$-bromoacetate analog used in their studies (Fig. 1). This affinity labeling agent was shown to be specific for VDR_{nuc} in a variety of nuclear extracts [Ray et al., 1996]; however, no specificity was shown in cytosolic or membrane fractions. This alkylating agent can react with a variety of different nucleophiles including amino acids containing nucleophilic R-groups, phospholipid head groups, and other primary and secondary hydroxyls [Sweet and Murdock, 1987]. Therefore, the decreased labeling of AII, proposed to be competition, in the presence of either a large excess of 1,25-D_3 or exogenous Ca^{2+}, could be caused by quenching of the observed nonspecific, covalent [^{14}C]-bromoacetate-AII adduct by the A-ring secondary hydroxyls and shielding of the exposed, calcium chelating, aspartic/glutamic acid carboxylates, respectively. This hypothesis is further supported by experiments that showed AI cross reacted with the [^{14}C]-bromoacetate analog and the band density was diminished in the presence of excess cold 1,25-D_3, yet AI did not specifically bind [^{3}H]-1,25-D_3 [Baran et al., 2000b]. Therefore, experiments inferring an ablation of 1,25-D_3-mediated rapid effects after addition of AII antibody are likely due to inactivation of the ability of AII to form complexes with itself and other proteins, not the inability to specifically bind 1,25-D_3 in the presence of the antibody.

Even though AII can be found localized to the caveolae [Oh and Schnitzer, 2001; Sowa et al., 2001; Stan, 2002; Uittenbogaard et al., 2002] it is very unlikely that it specifically binds 1,25-D_3, given the data presented in this communication. However its known cellular localization and regulation [Glenney and Tack, 1985; Bellagamba et al., 1997; Delouche et al., 1997; Rothhut, 1997] infer that AII could be downstream of the secretion stimulus reported for 1,25-D_3 in ROS 17/2.8 cells [Zanello et al., 2002].

Undoubtedly AII does not function in all 1,25-D_3 rapid signaling pathways, for addition of either an N-terminal and C-terminal antibody to AII was shown to have no effect on 1,25-D_3 induced PKC activity in matrix vesicles isolated from growth zone chondrocytes [Schwartz et al., 2002].

The search for the putative membrane receptor(s) for 1,25-D_3 continues, but given the evidence presented here, AII can be ruled out. Further experimentation is needed to investigate AIIs possible transitory role in mediating some of the rapid effects elicited by 1,25-D_3. Thus we are currently continuing our efforts to biochemically characterize the binding protein for [^{3}H]1,25-D_3 present in rat and chick intestinal CMF. Also it will be essential to link this ligand binding protein with rapid signal transduction events, and it is possible that AII may be participating in some of these processes.

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