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Mechanisms Involved in Vitamin D Mediated Intestinal Calcium Absorption and in Non-Classical Actions of Vitamin D

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Abstract

Recent studies in our laboratory using calbindin-D_{9k} null mutant mice as well as mice lacking the 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) inducible epithelial calcium channel TRPV6 provide evidence for calbindin-D_{9k} and TRPV6 independent regulation of active intestinal calcium absorption. These findings suggest that in the knock out (KO) mice there is compensation by another calcium channel or protein and that other novel factors are involved in 1,25$(OH)_2D_3$ mediated active intestinal calcium absorption. In addition, 1,25$(OH)_2D_3$ mediated paracellular transport of calcium may have contributed to the normalization of serum calcium in the null mutant mice. 1,25$(OH)_2D_3$ downregulates cadherin-17 and upregulates claudin-2 and claudin-12 in the intestine, suggesting that 1,25$(OH)_2D_3$, by regulating these epithelial cell junction proteins, can route calcium through the paracellular path. With regard to non-classical actions, 1,25$(OH)_2D_3$ has been reported to inhibit the proliferation of a number of malignant cells and to regulate adaptive as well as innate immunity. This article will review new developments related to the function and regulation of vitamin D target proteins in classical and non-classical vitamin D target tissues that have provided novel insight into mechanisms of vitamin D action.

1. Introduction

Studies in vitamin D receptor (VDR) knockout (KO) mice have indicated that the major role of 1,25$(OH)_2D_3$ is intestinal calcium transport [1,2]. However, the exact mechanisms involved in 1,25$(OH)_2D_3$ stimulation of intestinal calcium absorption remain to be defined. It has been proposed that the process of transcellular calcium transport involves apical entry of calcium via the apical calcium channel, transient receptor potential vanilloid type 6 (TRPV6), translocation of calcium through the interior of the enterocyte (it has been suggested that the calcium binding protein, calbindin-D_{9k} acts to facilitate calcium diffusion) and basolateral extrusion of calcium by the intestinal plasma membrane pump PMCA 1b [3]. Previous studies provided indirect evidence for a role of calbindin-D_{9k} and TRPV6 in intestinal calcium absorption. Calbindin-D_{9k} and TRPV6 are colocalized in the duodenum and jejunum and are similarly regulated [both are induced at weaning (the time of onset of active intestinal calcium transport), under conditions of low dietary calcium and

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after $1,25(OH)_2D_3$ injection] [4]. In addition, TRPV6 mRNA and calbindin-D_{9k} are induced by $1,25(OH)_2D_3$ in the intestine prior to the peak of intestinal calcium absorption [4]. However, the exact in vivo function of TRPV6 and calbindin-D_{9k} has been a matter of debate. The generation of TRPV6 and calbindin-D_{9k} KO mice made possible for the first time in vivo studies of the role of TRPV6 and calbindin-D_{9k} in $1,25(OH)_2D_3$ regulated intestinal calcium absorption [5,6].

With regard to non-classical actions of $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ has been reported to inhibit the proliferation of a number of malignant cells including breast cancer cells and to have immunosuppressive effects. The molecular mechanisms of $1,25(OH)_2D_3$ action are only now beginning to be defined. This article focuses on research from our laboratory related to an understanding of the function of vitamin D target proteins and the mechanisms of $1,25(OH)_2D_3$ action in classical and non-classical vitamin D target tissues.

2. Materials and Methods

TRPV6 KO mice and calbindin-D_{9k} KO mice were generated as previously described by Bianco et al [5] and Lee et al [6] respectively. TRPV6/calbindin-D_{9k} double KO (DKO) mice were generated in our laboratory by breeding TRPV6 KO females with calbindin-D_{9k} KO male mice for generation of double heterozygote mice which were subsequently bred to obtain TRPV6/calbindin-D_{9k} DKO mice. Intestinal calcium transport was determined by the everted gut sac assay [7]. Serum concentrations of calcium were determined using Sigma diagnostic reagents. Serum intact PTH levels were measured using the two site immunoradiometric assay (Immunotopics, San Clemente, CA). For transcription assays using the IL-17 promoter, the Jurkat human T cells line (from ATCC) was used. The human IL-17 promoter (−1124/+5) and deletion constructs were obtained from Sarah Gaffen (U. of Pittsburgh). For transcription assays using the cathelicidin promoter, A549 human lung epithelial cells were used. The human cathelicidin antimicrobial peptide gene promoter was obtained from Drs. P. Koeffler and A. Gombert. For transcription assays using the human cadherin-17 promoter (−1000/+48 from Eric Fearon, U. of Michigan Medical School) COS-7 cells were used. Transcription assays were performed by standard protocols [8,9]. C/EBP α expression vector was a gift of Simon Williams (Texas Tech University, Lubbock, TX). C/EBPα, VDR, cadherin-17 and NFAT antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Chromatin immunoprecipitation assays were performed as described earlier [8,9].

3. Results and discussion

3.1. Intestine: Intestinal calcium transport in the absence of TRPV6 and calbindin-D_{9k}

When fed a standard rodent chow diet, TRPV6 KO, calbindin-D_{9k} KO and TRPV6/calbindin-D_{9k} DKO mice were found to have serum calcium levels similar to those of WT mice (Fig. 1A, upper panel) [10]. In the TRPV6 KO and the TRPV6/calbindin-D_{9k} DKO mice serum PTH levels were significantly increased compared to WT (Fig. 1A, lower panel) [10]. The increase in PTH is consistent with a 9.6% decrease in femoral bone density previously reported in the TRPV6 KO mice and suggests that TRPV6 may have an indirect role in regulating bone formation and/or mineralization [5]. Active intestinal calcium transport in these mice was assessed using the everted gut sac assay. A significant two fold increase in active intestinal calcium transport was observed in vitamin D deficient WT, TPRV6 KO and calbindin-D_{9k} KO mice after $1,25(OH)_2D_3$ administration [no significant difference among these groups ($p > 0.1$)]. In the TRPV6/calbindin-D_{9k} DKO mice there was a 1.4 fold induction in active calcium transport after $1,25(OH)_2D_3$ administration (Fig. 1B; $p < 0.05$ compared to calcium transport in WT and single KO mice injected with $1,25(OH)_2D_3$), suggesting that the response to $1,25(OH)_2D_3$ may be more sensitive to the lack of both TRPV6 and calbindin than to the absence of either TRPV6 or calbindin alone [10]. These findings also suggest that in the KO mice there is compensation by another calcium channel or protein and that other factors involved in 1,25$(OH)_2D_3$ mediated intestinal calcium absorption remain to be identified. It is possible that 1,25$(OH)_2D_3$ mediated paracellular transport of calcium may have contributed to the normalization of serum calcium in the nullmutant mice. Recent studies have shown that 1,25$(OH)_2D_3$ can regulate tight junction and transmembrane proteins in the intestine including caludin-2, claudin-12 and cadherin-17 (the cadherin expressed in humans and mice exclusively in intestine and colon), suggesting an additional role for 1,25$(OH)_2D_3$ in the transjunctional movement of calcium [11,12]. Studies in our lab have indicated a significant decrease in cadherin-17 expression in duodenum under low dietary calcium conditions and in Caco-2 cells after 1,25$(OH)_2D_3$ treatment (Fig. 2). In addition, 1,25$(OH)_2D_3$/VDR can inhibit the induction of cadherin-17 transcription by the intestine specific transcription factor Cdx2 (Fig. 2). Thus, although it has been a matter of debate, these studies suggest that 1,25$(OH)_2D_3$ does in fact affect paracellular transport of the intestinal epithelium. Other possible factors involved in intestinal calcium transport include the L type calcium channel isoform Cav1.3 present in highest concentrations in the jejunum and ileum [13] and the calcium binding protein sorcin, which is induced by 1,25$(OH)_2D_3$ [14], has been reported to bind and modulate L type calcium channels [15] and is also present in highest concentrations in jejunum and ileum (Ajibade and Christakos, unpublished). Further studies are needed examining different regions of the intestine (not only duodenum) as well as novel 1,25$(OH)_2D_3$ regulated proteins involved in both transcellular and paracellular transport in order to provide new insight into the major role of 1,25$(OH)_2D_3$ in intestinal calcium absorption.

3.2. Non-classical actions of 1,25$(OH)_2D_3$

With regard to non-classical actions of 1,25$(OH)_2D_3$, numerous studies have shown that 1,25$(OH)_2D_3$ can exert inhibitory effects on the growth of a number of malignant cells, including breast cancer cells. The molecular mechanisms are now beginning to be defined. Recent studies in our lab have shown that C/EBP α, considered a potential tumor suppressor gene in breast cancer, is induced by 1,25$(OH)_2$D3 in MCF-7 breast cancer cells, cooperates with Brm (an ATPase that is a component of the SWI/SNF complex) and enhances VDR transcription [16]. Since levels of VDR correlate with the antiproliferative effects of 1,25$(OH)_2D_3$ and since 1,25$(OH)_2D_3$ and C/EBP α upregulate p21 (that functions as a regulator of cell cycle progression), these findings suggest mechanisms whereby 1,25$(OH)_2D_3$ acts to inhibit the growth of breast cancer cells. These findings also provide evidence for C/EBP α as a candidate for breast cancer treatment.

Additional non-classical actions of 1,25$(OH)_2D_3$ include effects on the immune system. 1,25$(OH)_2D_3$ has immunosuppressive effects which are correlated with a decrease in IL-2, IFN γ and GM-CSF [17]. Recent studies in our lab, in collaboration with the L. Steinman lab (Stanford University) have indicated that 1,25$(OH)_2D_3$ has a direct repressive effect on the expression of IL-17, a cytokine that has been reported to play a role in the pathogenesis of autoimmune inflammation [18]. The mechanism involves, at least in part, a competition of VDR with NFAT for binding to the NFAT element (Fig. 3). 1,25$(OH)_2D_3$ not only regulates adaptive but also innate immunity. 1,25$(OH)_2D_3$ induces the antimicrobial peptide cathelicidin with subsequent killing of bacteria including pseudomonas aeruginosa, a pathogen of lung infection in cystic fibrosis [19]. Recently we found that C/EBP α is a potent enhancer of cathelicidin antimicrobial peptide (CAMP) gene transcription and that C/EBP α functionally cooperates with VDR in the regulation of CAMP transcription (Fig. 4). Thus, there is increasing evidence that C/EBP isoforms may be key mediators of

$1,25(OH)_2D_3$ action in different cells. Further studies are needed examining global networks regulated by VDR to promote the host response to pathogen.

In conclusion, identification of target proteins as well as studies related to mechanisms of $1,25(OH)_2D_3$ action, including genome-wide action, will result in new insight in both classical and non-classical actions of vitamin D that may suggest therapeutic targets.

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References

1. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, Demay MB. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. Proc. Natl. Acad Sci. U. S. A 1997;94:9831–9835. [PubMed: 9275211]
2. Yoshizawa T, Handa Y, Uematsu Y, Takeda S, Sekine K, Yoshihara Y, Kawakami T, Arioka K, Sato H, Uchiyama Y, Masushige S, Fukamizu A, Matsumoto T, Kato S. Mice lacking the vitamin D receptor exhibit impaired bone formation, uterine hypoplasia and growth retardation after weaning. Nat. Genet 1997;16:391–396. [PubMed: 9241280]
3. Wasserman, RH. Vitamin D and the intestinal absorption of calcium: a view and overview. In: Feldman, D.; Pike, JW.; Glorieux, F., editors. Vitamin D. San Diego, CA: Elsevier Academic Press; 2005. p. 411-428.
4. Song Y, Peng X, Porta A, Takanaga H, Peng J-B, Hediger MA, Fleet J, Christakos S. Calcium transporter 1 and epithelial calcium channel messenger ribonucleic acid are differentially regulated by 1,25dihydroxyvitamin D3 in the intestine and kidney of mice. Endocrinology 2003;144:3885–3894. [PubMed: 12933662]
5. Bianco SD, Peng J-B, Takanaga H, Suzuki Y, Crescenzi A, Kos CH, Zhuang L, Freeman MR, Gouveia CH, Wu J, Luo H, Mauro T, Brown EM, Hediger MA. Marked disturbance of calcium homeostasis in mice with targeted disruption of the TRPV6 calcium channel gene. J. Bone Miner. Res 2007;22:274–285. [PubMed: 17129178]
6. Lee GS, Lee KY, Choi KC, Ryu YH, Paik SG, Oh GT, Jeung EB. A phenotype of a calbindin-D9k gene-knockout is compensated for by the induction of other calcium transporter genes in a mouse model. J. Bone Miner. Res 2007;22:1968–1978. [PubMed: 17696760]
7. Armbrecht HJ, Boltz MA, Hodam TL. Differences in intestinal calcium and phosphate transport between low and high bone density mice. Am. J. Physiol. Gastrointest. Liver Physiol 2002;282:G130–G136. [PubMed: 11751166]
8. Shen Q, Christakos S. The vitamin D receptor, Runx2 and the notch signaling pathway cooperate in the transcriptional regulation of osteopontin. J. Biol. Chem 2005;280:40589–40598. [PubMed: 16195230]
9. Zhong Y, Armbrecht HJ, Christakos S. Calcitonin: a regulator of 25-hydroxyvitamin D3 1 α hydroxylase gene. J. Biol. Chem 2009;284:1159–1169.
10. Benn BS, Ajibade D, Porta A, Dhawan P, Hediger M, Peng J-B, Jiang Y, Oh GT, Jeung EB, Lieben L, Bouillon R, Carmeliet G, Christakos S. Active intestinal calcium transport in the absence of transient receptor potential vanilloid type 6 and calbindin-D_{9k}. Endocrinology 2008;149:3196–3205. [PubMed: 18325990]
11. Kutuzova GD, DeLuca HF. Gene expression profiles in rat intestine identify pathways for 1,25-dihydroxyvitamin D3 stimulated calcium absorption and clarify its immunomodulatory properties. Arch. Biochem. Biophys 2004;432:152–166. [PubMed: 15542054]
12. Fujita H, Sugimoto K, Inatomi S, Maeda T, Osanai M, Uchiyama Y, Wada T, Kojima T, Yokozaki H, Yamashita T, Kato S, Sawada N, Chiba H. Tight junction proteins claudin-2 and -12 are critical for vitamin D-dependent Ca^{2+} absorption between enterocytes. Molecular Biol. Cell 2008;19:1912–1921.

13. Morgan EL, Mace OJ, Helliwell PA, Affleck J, Kellett GL. A role for Cav1.3 in rat intestinal calcium absorption. Biochem. Biophys. Res. Commun 2003;312:487–493. [PubMed: 14637163]

14. Wood RJ, Tchack L, Angelo G, Pratt RE, Sonna LA. DNA microarray analysis of vitamin D-induced gene expression in a human colon carcinoma cell line. Physiol. Genomics 2004;17:122–129. [PubMed: 14996990]

15. Meyers MB, Puri TS, chien AJ, Gao T, Hsu PH, Hosey MM, Fishman GI. Sorcin associates with pore-forming subunit of voltage-dependent L-type Ca2+ channels. J. Biol. Chem 1998;273:18930–18935. [PubMed: 9668070]

16. Dhawan P, Wieder R, Christakos S. CCAAT enhancer binding protein alpha is a molecular target of 1,25-dihydroxyvitamin D3 in MCF-7 breast cancer cells. J. Biol. Chem 2009;284:3086–3095. [PubMed: 19054766]

17. Raghuwanshi A, Joshi S, Christakos S. Vitamin D and multiple sclerosis. J Cell. Biochem 2008;105:338–343. [PubMed: 18655192]

18. Joshi S, Youssef S, Gaffen S, Steinman L, Christakos S. A key mechanism underlying the immunosuppressive effects of vitamin D: 1,25dihydroxyvitamin D3 is a transcriptional modulator of IL-17. J. Bone Mineral Res 2008;23:S105.

19. Yim S, Dhawan P, Ragunath C, Christakos S, Diamond G. Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D3. J. Cyst. Fibros 2007;6:403–410. [PubMed: 17467345]

20. Hinoi T, Lucas PC, Kuick R, Hanash S, Cho KR, Fearon ER. Cdx2 regulates liver intestine cadherin expression in normal and malignant colon epithelium and intestinal metaplasia. Gastroenterology 2002;123:1565–1577. [PubMed: 12404231]

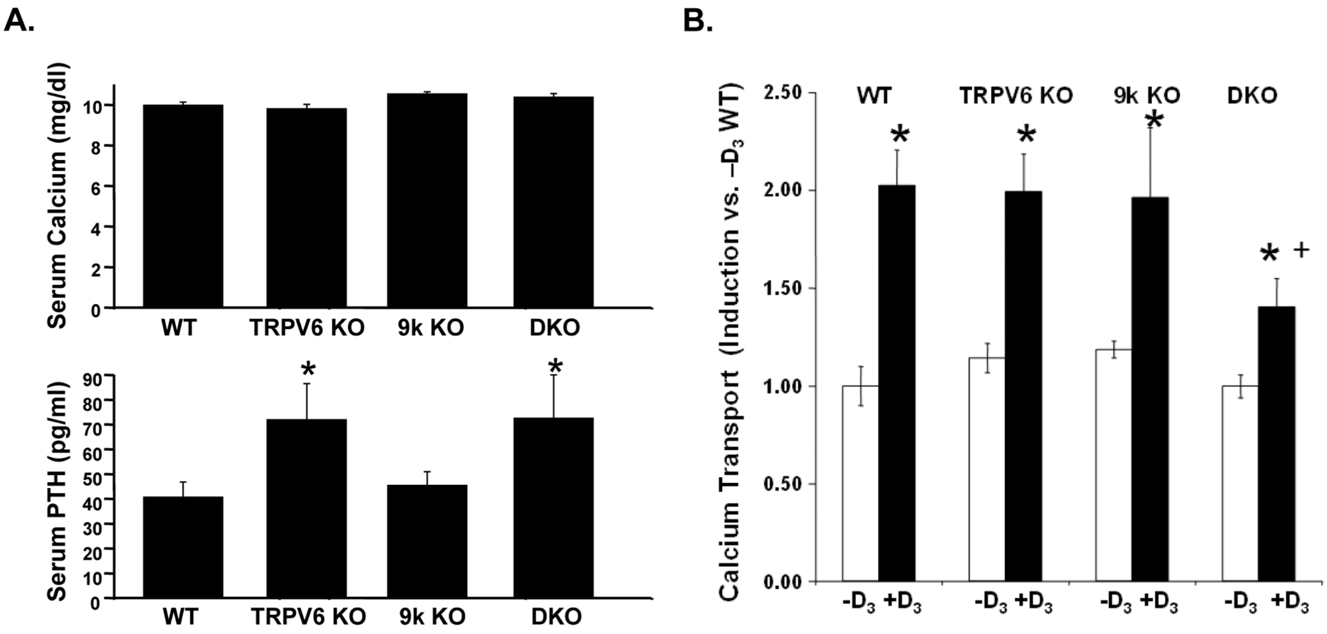

Fig 1.

Serum calcium and PTH and $1,25(OH)_2D_3$ stimulated duodenal calcium transport in WT and nullmutant mice. A. Each value for serum calcium or PTH represents the mean +/− SEM for male mice (n = 7 – 23 mice per group; *, p <0.05 compared to WT. B. Calcium transport was measured using everted intestinal sacs from the duodenum of 12-wk old mice made $1,25(OH)_2D_3$ deplete by feeding a 0.8% strontium diet for 7 days. Mice were then injected 3 times with $1,25(OH)_2D_3$ (+D) or vehicle (−D) 48, 24 and 6 h before termination (ip, 100 ng/100g body weight/injection). Values represent the mean +/− SEM (n = 6 – 16/ group; *, p < 0.05 for $1,25(OH)_2D_3$ treated (+D) compared with the respective deficient (−D) mice; +, p <0.05 compared with WT +D).

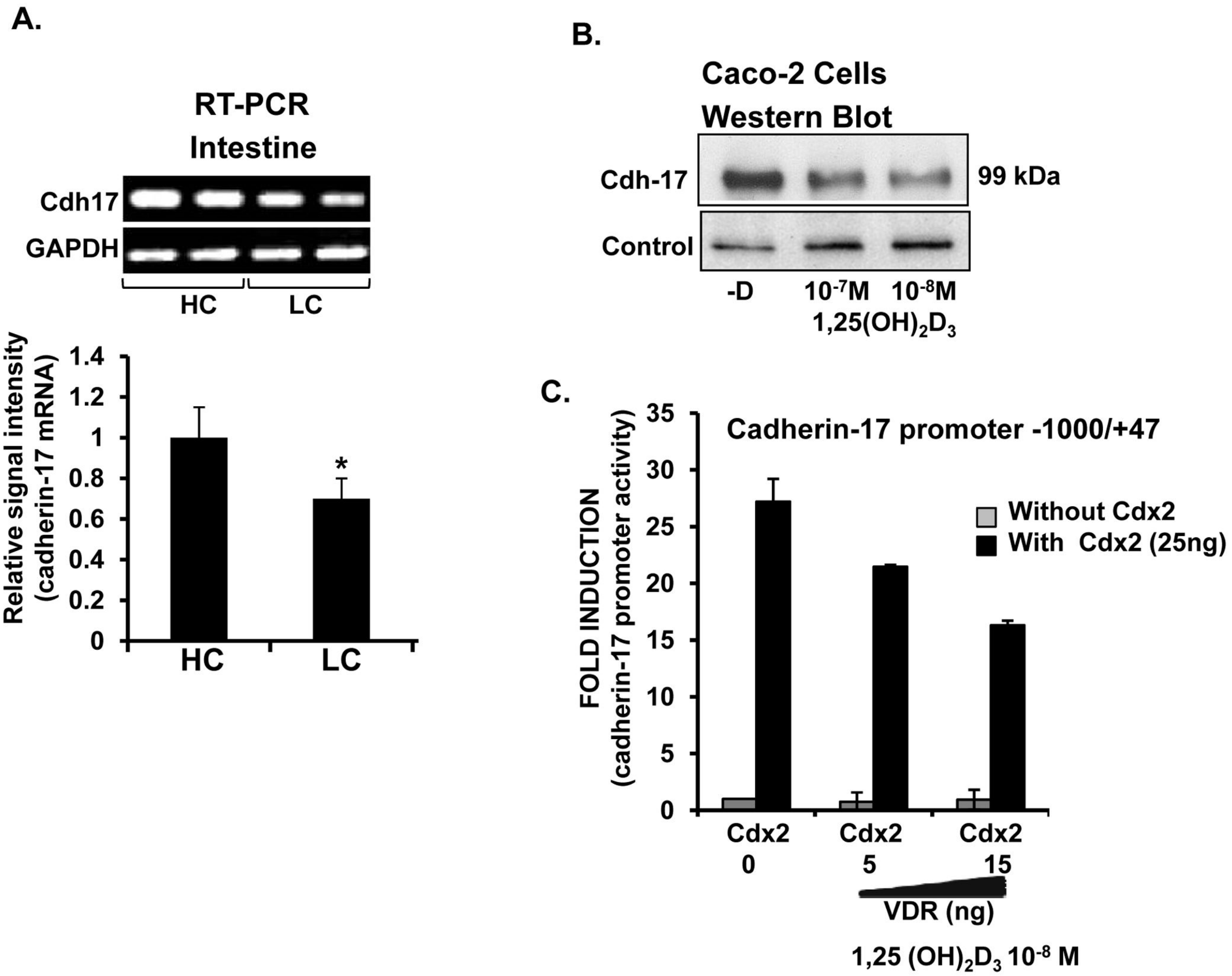

Fig 2.

Inhibition of cadherin-17 under low dietary calcium conditions and by $1,25(OH)_2D_3$. A. Two month old mice were fed a high calcium (HC, 1%) calcium or low (LC, 0.02%) calcium diet from 4 weeks of age. RNA was prepared from the duodenum, reverse transcribed and subjected to PCR with primers to amplify the appropriate DNA fragment of cadherin-17 (20). * $p < 0.05$ compared to HC. Note a decrease in duodenal cadherin-17 under low dietary calcium conditions (a 4 fold induction in $1,25(OH)_2D_3$ serum levels were noted in the mice fed the LC diet). B. Cadherin-17 is inhibited in Caco-2 cells by $1,25(OH)_2D_3$. Representative Western blot of cadherin-17 expression in Caco-2 cells treated with vehicle (−D) or $1,25(OH)_2D_3$ for 24h. C. Cdx2 induced transcription of cadherin-17 is inhibited by $1,25(OH)_2D_3$. COS-7 cells were transfected with Cdx2 expression vector (15ng) in the presence or absence of VDR. Cells were treated with vehicle or 1,25(OH)2D3 (10-8M) for 24h and luciferase activity was determined. The data were normalized to values for pRL-TK Renilla luciferase as an internal control. Cadherin-17 promoter activity is represented as fold induction (mean + SE, n= 3 observations) and quantified by comparison to basal levels.

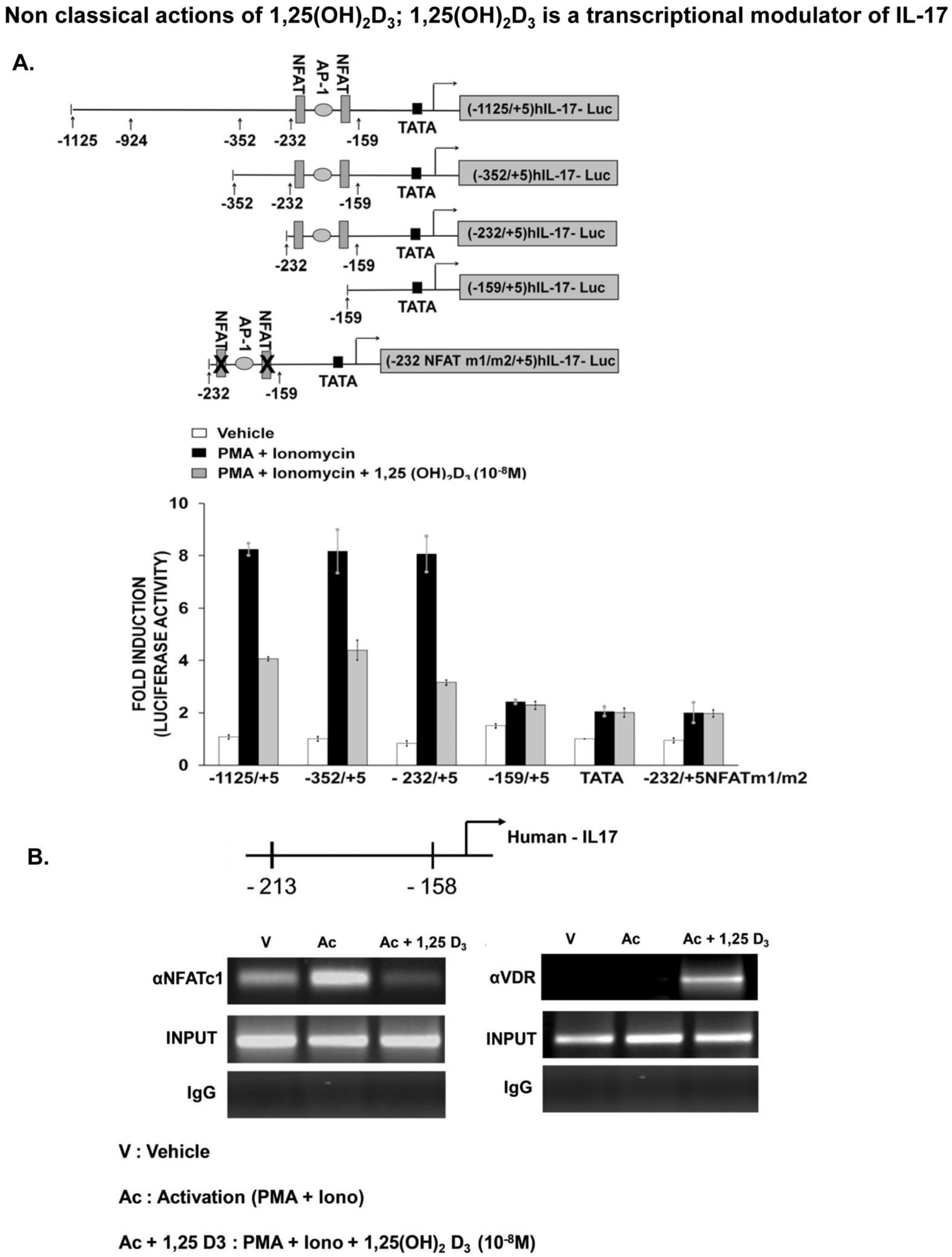

Fig 3.

Inhibition of hIL-17 promoter activity by $1,25(OH)_2D_3$ and recruitment of NFATc1 and VDR by ChIP assay on the hIL-17 promoter. A. The human T cell line Jurkat was cotransfected with the hIL-17 promoter or deletion/mutation promoter constructs and VDR expression vector. Cell were incubated with PMA and ionomycin for 8h in the presence of absence of $1,25(OH)_2D_3$. Note, mutation of the NFAT sites (and not the AP1site) inhibits activation and inhibition by $1,25(OH)_2D_3$. Using the −232/+5 construct, co-transfection with NFATc1 expression vector reversed the inhibition observed with $1,25(OH)_2D_3$ (not shown). B. For ChIP assays HUT102 cells were used which constitutively produce IL-17. HUT cells transfected with VDR were treated with vehicle, PMA + ionomycin or PMA + ionomycin

+1,25(OH)$_2$D$_3$ for 1h and cross-linked by 1% formaldehyde for 15 min. Cross linked cell lysates were subjected to immunoprecipitation with NFATc1 antibody or VDR antibody. DNA precipitates were isolated and then subjected to PCR using specific primers designed to the −213/−158 region of the hIL-17 promoter which contains NFAT sites. Note ChIP assay shows that in the presence of 1,25(OH)$_2$D$_3$ and VDR recruitment of NFATc1 was inhibited and recruitment of VDR to this site is now observed, suggesting that blocking of the NFAT site by VDR is one mechanism involved in 1,25(OH)$_2$D$_3$/VDR mediated inhibition of IL-17.

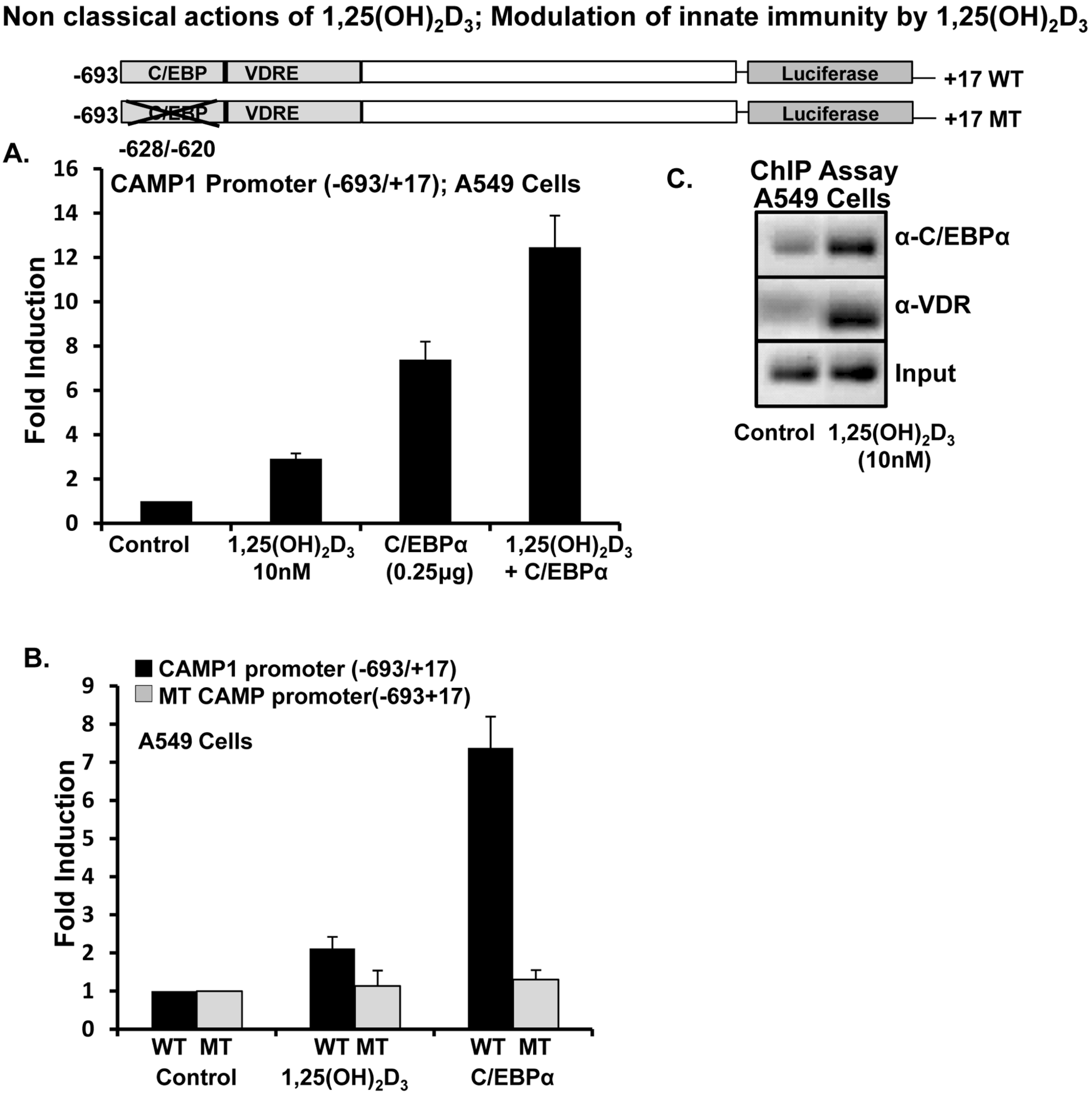

Fig 4.

Activation of the cathelicidin antimicrobial peptide (CAMP) gene promoter by $1,25(OH)_2D_3$ and C/EBP α and recruitment of C/EBP α and VDR by ChIP assay on the CAMP promoter. A. and B. Human lung epithelial cells A549 were transfected with the CAMP promoter or with the CAMP promoter with the putative C/EBP site (at −628/−620) mutated (MT CAMP promoter, B). Note, mutation of the C/EBP site attenuates the transcriptional response to C/EBP α as well as to $1,25(OH)_2D_3$ (*, $p < 0.05$ compared to WT). C. ChIP analysis using specific primers designed to include both the C/EBP site (−628/−620) and the adjacent VDRE (−616/−601) shows C/EBP α and VDR recruitment to the CAMP promoter by $1,25(OH)_2D_3$ in A549 cells.