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Response element binding proteins and intracellular vitamin D binding proteins: novel regulators of vitamin D trafficking, action and metabolism☆

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Abstract

Using vitamin D-resistant New World primates as model of natural diversity for sterol/steroid action and metabolism, two families of novel intracellular vitamin D regulatory proteins have been discovered and their human homologs elucidated. The first family of proteins, heterogeneous nuclear ribonucleoproteins (hnRNPs), initially considered to function only as pre-mRNA-interacting proteins, have been demonstrated to be potent *cis*-acting, *trans*-dominant regulators of vitamin D hormone-driven gene transactivation. The second group of proteins bind 25-hydroxylated vitamin D metabolites. Their overexpression increases vitamin D receptor (VDR)-directed target gene expression. We found that these intracellular vitamin D binding proteins (IDBPs) are homologous to proteins in the heat shock protein-70 family. Our ongoing studies indicate directly or indirectly through a series of protein interactions that the IDBPs interact with hydroxylated vitamin D metabolites and facilitate their intracellular targeting.
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1. In the beginning

In the Eocene period, some 50–100 million years ago, the great southern hemispheric land mass, Pangea, broke apart with the Americas and Madagascar moving away from Africa. This continental separation occurred early in the process of primate evolution, trapping primordial primates in South America, Africa, and Madagascar. Because they lacked aquatic mobility, the three major primate infraorders, platyrrhines or New World primates, the catarrhines or Old World primates and lemurs (i.e. prosimians), respectively, evolved independently of one another on these three separate land masses [1]. Compared to Old World primates, including Homo sapiens which exhibit a worldwide distribution today, New World primates are confined to South and Central America roughly 20° latitude north and south of the equator. They are generally smaller in stature which is well suited to their lifestyle as herbivorous sunbathers, residing in the canopy of the periequatorial rain forests of the Americas.

2. Lessons learned from an outbreak of rickets in the New World primate colonies of the Los Angeles Zoo

In the mid-1980s, the authors were asked to investigate the cause and provide a treatment for a rachitic bone disease that was rampant among pre-adolescent New World primate residents of the Los Angeles Zoo. The disorder was most severe among monkeys of the Emperor tamarin species. When investigated radiographically, affected monkeys displayed classical rickets. Compared to unaffected age-matched siblings, these animals had smaller skeletons and metaphyseal cupping and fraying characteristic of rickets.

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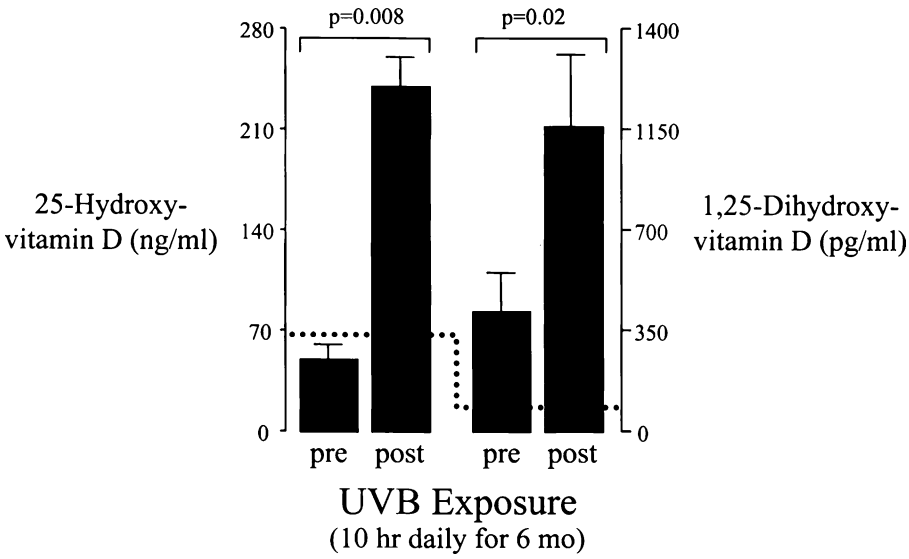


Fig. 1. Mean serum levels of 25-(OH)D on the left and 1,25-$(OH)_2$D levels on the right in seven different rachitic New World primates before and after exposure to 6 months of artificial sunlight in their enclosures. The upper limits of the normal human Old World primate range is described by the dotted line. Both substrate and product rose dramatically with light therapy, an increase that resulted in cure of rickets.

When compared to control, nonrachitic, age- and sex-matched New and Old World primates from a variety of different genera, rachitic New World primates displayed a serum and urine biochemical phenotype that was remarkable for [1] a slightly decreased serum calcium [2], a slightly decreased fractional urinary calcium excretion rate and [3] an elevated serum 1,25-$(OH)_2$D level [2]. With the exception of nocturnal primates in the genus Aotus, New World primates in all other genera had hormone levels ranging up to as high as two orders of magnitude greater than that observed in Old World primates, including man [2–4]. The New World primates with rickets were those with the lowest 1,25-$(OH)_2$D levels. These data were interpreted to mean that most New World primate genera were naturally resistant to the vitamin D hormone and that this resistant state could be successfully compensated by maintenance of high circulating 1,25-$(OH)_2$D levels. Proof of this fact is provided in Fig. 1 which shows serum substrate 25-(OH)D and product 1,25-$(OH)_2$D concentrations in seven individual rachitic New World primates before and after exposure to 6 months of artificial sunlight in their enclosures [5]. Both substrate 25-(OH)D and product 1,25-$(OH)_2$D rose significantly with light therapy resulting in cure of rickets, and led to the conclusion that New World primates are periequatorial sunbathers for a reason. They require substantial levels of cutaneous vitamin D synthesis in order to push their 25-(OH)D and 1,25-$(OH)_2$D levels high enough to effectively interact with the vitamin D receptor (VDR). In their native environment $\pm 20^\circ$ about the equator, these monkeys can make vitamin D in their skin year-round. However, when transported by humans to an unnatural habitat at 40° North latitude in Los Angeles, they were no longer able to sustain adequate 1,25-$(OH)_2$D hormone synthesis.

3. Vitamin D response element binding proteins as the cause of vitamin D resistance in New World primates

In order to answer the question as to why New World primates were resistant to all but the highest levels of the vitamin D hormone, cultured fibroblasts and immortalized cell lines from resistant- and hormone-responsive New and Old World primates, respectively, were employed to investigate, step by step, the path taken by the vitamin D substrate and hormone from the serum vitamin D binding protein (DBP) in the blood in route to the nucleus and transactivation of hormone-responsive genes. It was discovered that the movement of 25-hydroxylated vitamin D_2 and D_3 metabolites from DBP, across the cell membrane, through the cytoplasm and through nuclear membrane into the nucleus in hormone-resistant New World primate cells was indistinguishable from that seen in hormone-responsive Old World primate cells; it was similarly observed that the ability of the New World primate VDR to bind 1,25-$(OH)_2$D and induce receptor dimerization with the retinoid X receptor (RXR) was normal [2,4–12]. In fact, when removed from the intranuclear environment, the VDR in New World primate cells was like the Old World primate VDR in all respects [13].

What was not the same in New World primate cells, was the reduced ability of VDR–RXR complex to bind to its cognate *cis* element and reduced potency to transactivate target genes. In order to determine the *cis–trans* interaction disturbance in the New World primate nucleus, the nuclei of New World primate cells were isolated and their protein constituents extracted. A second set of proteins, in addition to the VDR–RXR complex, that could be specifically bound by the vitamin D response element (VDRE) were found to

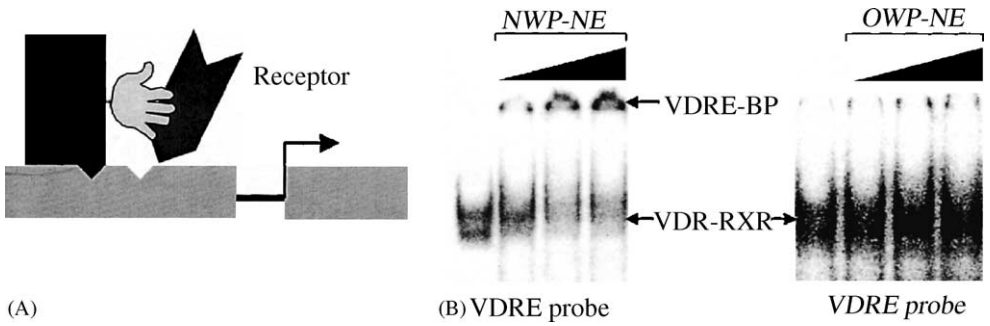


Fig. 2. Proposed "dominant-negative" action of the New World primate vitamin response element binding protein (VDRE-BP) to compete receptor away from the vitamin D response element (VDRE; panel A). In electrophoretic mobility shift analysis (panel B) recombinant human vitamin D receptor (VDR) and retinoid X receptor (RXR) were permitted to interact with VDRE probe in the presence of increasing amounts of nuclear extract from vitamin D-resistant New World primate (NWP) cells containing VDRE-BP on the left or from vitamin D-responsive Old World primate (OWP) cells on the right. Adding more control extract only served to amplify the VDR–RXR-retarded probe in the gel. By contrast, adding increasing concentrations the hormone-resistant NWP extract competed away VDR–RXR binding in favor of VDRE-BP-probe binding.

be present and overexpressed in New World primate cell nuclei [12]. As shown in panel A of Fig. 2, these data suggested that this vitamin D response element binding protein(s) (VDRE-BP) might function as a dominant-negative inhibitor of receptor-response element binding by competing in *trans* with receptor, "knocking it off" the VDRE. That appears to be the case (panel B, Fig. 2). In electrophoretic mobility shift analyses (EMSA), recombinant human VDR and RXR were competed away from a wild-type, consensus VDRE probe in the presence of increasing amounts of nuclear extract from vitamin D-resistant New World primate cells containing VDRE-BP(s) but not from Old World primate cells.

VDRE (DNA)-affinity chromatography has now been employed to identify three VDRE-BPs. All are members of the heterogeneous nuclear ribonucleoproteins-A (hnRNPA) family, previously considered to be only single strand RNA binding proteins [14]. However, these proteins can also bind specifically to double strand DNA, a fact that distinguishes them from traditional co-repressor proteins [15,16]. When over-expressed in vitro, they can effectively squelch transactivation [12]; VDRE-BP2, the most potent of the VDRE-BPs, reproducibly squelched VDRE-directed transactivation by ≥80% when stably overexpressed in wild-type, vitamin D-responsive Old World primate cells.

4. Discovery of a human homolog of the vitamin D response element binding protein

When overexpressed in vivo, these VDRE-BPs can cause rickets in sunlight-deprived New World primates, and there is now evidence that they can do the same in an adolescent human. Over a decade ago, Hewison et al. [17,18] described a young girl, an only child of two phenotypically normal parents, with VDR- and RXR-normal vitamin D-resistant rickets and alopecia. Using primary cultures of dermal fibroblasts from the patient in a VDR-VDRE-directed luciferase-reporter assay and compared to cells from an age-matched control, reporter activity was remarkably diminished, barely stimulated with the addition of exogenous vitamin D hormone, and not rescued by co-transfection of additional VDR. Like the vitamin D-resistant New World primates, this girl's cells harbored a response element binding protein or REBiP (panel A, Fig. 3) [19]. REBiP is a

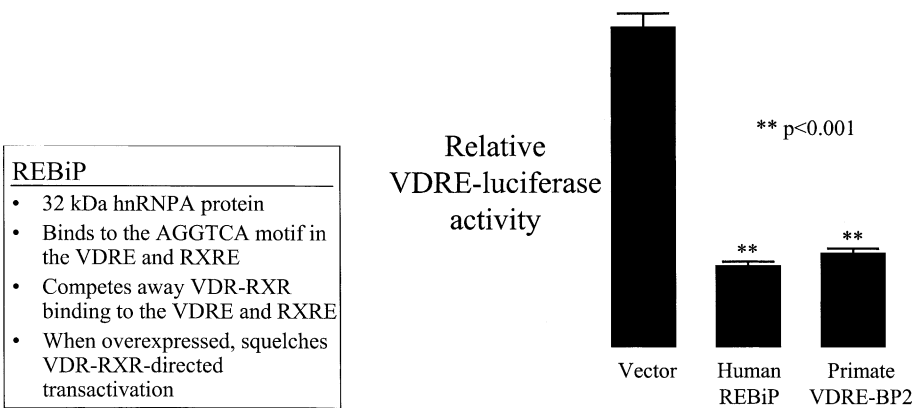
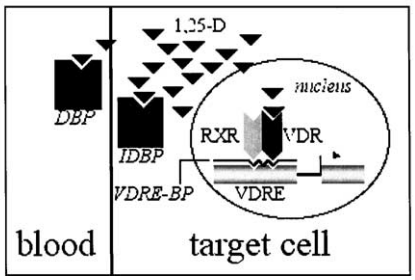


Fig. 3. Characteristics of the human response element binding protein (REBiP; panel A). Panel B demonstrates the ability of the transfected REBiP and New World primate VDRE-BP2 to significantly squelch vitamin D response element (VDRE)-directed transactivation in a luciferase reporter assay.

(A)



(B)

NWP IDBP	human hsp homolog	sequence identity
IDBP-1	hsc70 [cyto]	95%
IDBP-2	grp78 [e.r.]	92%
IDBP-3	grp75 [mito]	92%
IDBP-4	hsp70 [cyto]	95%

Fig. 4. Schematic representation of the state of 1,25-$(OH)_2$D (1,25-D) resistance in the New World primate target cell bearing (panel A): a relative excess of intracellular vitamin D binding protein(s) (IDBP); a relative excess of internalized hormone; the vitamin D receptor (VDR) and retinoid X receptor (RXR); a vitamin D response element (VDRE)-regulated gene; the dominant-negative-acting vitamin D response element binding protein (VDRE-BP);. Also depicted is the serum vitamin D binding protein (DBP), which transports hormone in the circulation. Panel B depicts the four known IDBPs so far isolated from vitamin D-resistant New World primate (NWP) cells and their human heat shock protein-70 (hsp) homologs; bracketed is the predominant intracellular site of residence for the human hsp.

32 kDa protein in the hnRNPA family which will bind to the AGGTCA motif of the VDRE as well as the RXRE [19]. Also like the VDRE-BPs from New World primates, when over-expressed REBiP will squelch VDR–RXR-directed transactivation (panel B, Fig. 3) [19]. In summary, vitamin D resistance in New World primates, as well as in at least a single human, results from the constitutive over-expression of at least one and possibly more than one hnRNP-related response element binding proteins. These proteins act in *trans* to block the ability of receptor dimers to interact with their respective response elements (panel A, Fig. 4).

5. Discovery of a family of intracellular vitamin D binding proteins in New World primate cells

During the investigation of the VDRE-BPs in New World primate cells, it was observed that these cells were extraordinarily efficient at accumulating 25-D-hydroxylated vitamin D metabolites in the cytoplasmic space (panel A, Fig. 4). Accumulation of metabolite here is the result of expression of a second set of resistance-associated proteins [20,21]. Using affinity chromatography, four of these intracellular vitamin D binding proteins (IDBPs) have been isolated from the post-nuclear extracts of New World primate cells. All four exhibit both high capacity and relatively high affinity (0.5–5.0 nM) for 25-hydroxylated vitamin D metabolites. Although present in Old World primate cells, including human cells, these proteins can be over-expressed $\geq$50-fold in New World primate cells. They are highly homologous to one another and to proteins in the human heat shock protein-70 family (panel B, Fig. 4). Primate IDBP-1, -2, -3, and -4 bear a high degree of sequence identity with the cytoplasmic constitutively-expressed human heat shock protein-70, the endoplasmic reticulum resident protein grp78, mitochondrial-targeted grp-75 and the largely cytoplasmic heat shock inducible heat shock protein-70, respectively. Structurally, all of the IDBPs (Fig. 4) contain an ATP-binding-ATPase domain ahead of a protein–protein interaction domain; some, like grp-75 (IDBP-3), also harbor an N-terminal organelle-targeting domain [22]. Preliminary studies indicate a central portion of these proteins is involved in their vitamin D binding activity [23].

The next question to be considered by our laboratory was the function of these proteins in the hormone-resistant New world primate cell. Two countervailing hypotheses were considered. One was that these IDBPs were “sink” molecules that worked in cooperation with the VDRE-BPs to exert vitamin D resistance by disallowing access of the hormone to the VDR and the nucleus of the cell. The second, opposing hypothesis held that these were “swim” molecules, promoting the delivery of ligand to the vitamin D receptor, improving the ability of the VDR to dimerize and bind DNA, hence antagonizing the actions of the VDRE-BPs that are overexpressed in New World primate cells. In order to address this issue, the human hsc70-related New World primate IDBP-1 was stably over-expressed in wild-type Old World primate cells and demonstrated to impart significant protransactivating potential [23]. At least for the function of transactivation, it was concluded that hsc70-related IDBP-1 was a “swim” molecule for the vitamin D hormone, most likely by promoting the delivery of ligand 1,25-$(OH)_2$D to the VDR.

Taking into consideration the facts that New World primates are required to maintain very high serum levels of 1,25-$(OH)_2$D in order to avert rickets, the possibility was considered that the IDBPs, which are known to bind 25-(OH)D even better that 1,25-$(OH)_2$D [20,21], might also serve to promote the synthesis of the active vitamin D metabolite via stimulation of the 1-hydroxylation reaction. When human kidney cells expressing the vitamin D-1-hydroxylase were stably transfected with the hsc-70-related IDBP-1 and incubated with substrate 25-$(OH)D_3$, 1,25-$(OH)_2D_3$ production went up four- to eight-fold compared to cells transfected with vector alone [24]. This increase in specific 1-hydroxylase activity occurred independent of any change in expression of the 1-hydroxylase gene.

6. A role for members of the heat shock protein-70 family in intracellular vitamin D trafficking

It has been classically held that sterol/steroid hormones, by nature of their lipid solubility, squeeze through the plasma membrane of the target cell and "ping-pong" around the cell interior until they encounter another specific binding protein like the 1-hydroxylase in the mitochondria or the VDR in the nucleus or they find the plasma membrane again and leave the cell the same way they came in. Recent genetic studies using knockout mouse models demonstrated that receptor-mediated endocytosis plays an important role in the uptake of hydroxylated vitamin D metabolites in the kidney [25–27]. For example, low density lipoprotein (LDL) family members megalin and cubulin are essential for internalization of the hormone from the glomerular filtrate. The hormone–megalin complex then enters the lysosomal compartment, where the carrier is degraded. Since heat shock proteins are essential for endocytosis, they may be important for targeting the hormone to specific cellular compartments.

Our current collaborative efforts are directed towards elucidating this cytoplasmic targeting mechanism.

Acknowledgements

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References

[1] D. Pilbeam, The descent of hominoids and hominoids and hominids, Sci. Am. 250 (1984) 84–96.
[2] J.S. Adams, M.A. Gacad, A.J. Baker, B. Gonzales, R.K. Rude, Serum concentrations of 1,25-dihydroxyvitamin D in Platyrrhini and Catarrhini: a phylogenetic appraisal, Am. J. Primatol. 9 (1985) 219–224.
[3] J.S. Adams, M.A. Gacad, R.K. Rude, D.B. Endres, L.E. Mallette, Serum concentrations of immunoreactive parathyroid hormone in Platyrrhini and Catarrhini: a comparative analysis with three different antisera, Am. J. Primatol. 13 (1987) 425–433.
[4] M.A. Gacad, J.S. Adams, Evidence for endogenous blockage of cellular 1,25-dihydroxyvitamin D-receptor binding in New World primates, J. Clin. Invest. 87 (1991) 996–1001.
[5] M.A. Gacad, J.S. Adams, Influence of ultraviolet B radiation on vitamin D3 metabolism in vitamin D3-resistant New World primates, Am. J. Primatol. 28 (1992) 263–270.
[6] J.S. Adams, M.A. Gacad, A.J. Baker, G. Keuhn, R.K. Rude, Diminished internalization and action of 1,25-dihydroxyvitamin D in dermal fibroblasts cultured from New World primates, Endocrinology 116 (1985) 2523–2527.
[7] J.S. Adams, M.A. Gacad, Phenotypic diversity of the cellular 1,25-dihydroxyvitamin D-receptor interaction among different genera of New World primates, J. Clin. Endocrinol. Metab. 66 (1988) 224–229.
[8] M.A. Gacad, J.S. Adams, Specificity of steroid binding in New World primate cells with a vitamin D-resistant phenotype, Endocrinology 131 (1992) 2581–2587.
[9] M.A. Gacad, J.S. Adams, Identification of a competitive binding component in vitamin D-resistant New World primate cells with a low affinity but high capacity for 1,25-dihydroxyvitmain D3, J. Bone Miner. Res. 8 (1993) 27–35.
[10] J.E. Arbelle, H. Chen, M.A. Gacad, E.A. Allegretto, J.W. Pike, J.S. Adams, Inhibition of vitamin D receptor-retinoid X receptor-vitamin D response element complex formation by nuclear extracts of vitamin D-resistant New World primate cells, Endocrinology 137 (1996) 786–789.
[11] H. Chen, J.E. Arbelle, M.A. Gacad, E.A. Allegretto, J.S. Adams, Vitamin D and gonadal steroid-resistant New World primate cells express an intracellular protein which competes with the estrogen receptor for binding to the estrogen response element, J. Clin. Invest. 99 (1997) 769–775.
[12] H. Chen, B. Hu, E.A. Allegretto, J.S. Adams, The vitamin D response element binding proteins: novel dominant-negative-acting regulators of vitamin D-directed transactivation, J. Biol. Chem. 275 (2000) 35557–35564.
[13] R.F. Chun, H. Chen, L. Boldrick, C. Sweet, J.S. Adams, Cloning, sequencing and functional characterization of the vitamin D receptor in vitamin D-resistant New World primates, Am. J. Primatol. 54 (2001) 107–118.
[14] G. Dreyfuss, M.J. Matunis, S. Pinol-Roma, C.G. Burd, hnRNP proteins and the biogenesis of mRNA, Annu. Rev. Biochem. 62 (1993) 289–321.
[15] K.B. Horwitz, T.A. Jackson, D.L. Bain, J.K. Richer, G.S. Takomoto, L. Tung, Nuclear receptor coactivators and corepressors, Mol. Endocrinol. 10 (1996) 1167–1177.
[16] A. Aranda, A. Pascual, Nuclear hormone receptors and gene expression, Physiol. Rev. 81 (2001) 1269–1304.
[17] M. Hewison, A.R. Rut, K. Kristjansson, R.E. Walker, M.J. Dillon, M.R. Hughes, J.L. O'Riordan, Tissue resistance to 1,25-dihydroxyvitamin D without a mutation of the vitamin D receptor gene, Clin. Endocrinol. (Oxf.) 39 (1993) 663–670.
[18] M. Hewison, J.L. O'Riordan, Hormone-nuclear receptor interactions in health and disease. Vitamin D resistance, Baillieres Clin. Endocrinol. Metab. 8 (1994) 305–315.
[19] H. Chen, M. Hewison, B. Hu, J.S. Adams, Heterogeneous nuclear ribonucleoprotein (hnRNP)-binding to hormone response elements: a novel cause of vitamin D resistance, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 6109–6114.
[20] M.A. Gacad, T.R. LeBon, H. Chen, J.E. Arbelle, J.S. Adams, Functional characterization and purification of an intracellular vitamin D binding protein in vitamin D resistant New World primate cells: amino acid sequence homology with proteins in the hsp-70 family, J. Biol. Chem. 272 (1997) 8433–8440.
[21] M.A. Gacad, J.S. Adams, Proteins in the heat shock-70 family specifically bind 25-hydroxylated vitamin D metabolites and 17β-estradiol, J. Clin. Endocrinol. Metab. 83 (1998) 1264–1267.
[22] F.U. Hartl, Molecular chaperones in cellular protein folding, Nature 381 (1996) 571–579.
[23] S. Wu, S. Ren, M.A. Gacad, J.S. Adams, Intracellular vitamin D binding proteins: novel facilitators of vitamin D-directed transactivation, Mol. Endocrinol. 14 (2000) 1387–1397.
[24] S. Wu, R. Chun, S. Ren, H. Chen, J.S. Adams, Regulation of 1,25-dihydroxyvitamin D synthesis by intracellular vitamin D binding protien-1, Endocrinology 143 (2002) 4135.
[25] A. Nykjaer, D. Dragun, D. Walther, H. Vorum, C. Jacobsen, J. Herz, F. Melsen, E.I. Christensen, T.E. Willnow, An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3, Cell 96 (1999) 507–515.
[26] E.I. Christensen, T.E. Willnow, Essential role of megalin in renal proximal tubule for vitamin homeostasis, J. Am. Soc. Nephrol. 10 (1999) 2224–2236.
[27] T.E. Willnow, A. Nykjaer, Pathways for kidney-specific uptake of the steroid hormone 25-hydroxyvitamin D3, Curr. Opin. Lipidol. 13 (2002) 255–260.