Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Steroid Biochemistry & Molecular Biology 89–90 (2004) 281–285
The Journal of Steroid Biochemistry & Molecular Biology
www.elsevier.com/locate/jsbmb

Identification and characterization of 1,25D_3-membrane-associated rapid response, steroid (1,25D_3-MARRS) binding protein☆

Ilka Nemere[a], Susan E. Safford[b], Benjamin Rohe[c], Mary M. DeSouza[c,1], Mary C. Farach-Carson[c,*]

[a] *Nutrition and Food Sciences, Utah State University, Logan, UT, USA*
[b] *Biology Department, Lincoln University, Lincoln, PA, USA*
[c] *Department of Biological Sciences, 326 Wolf Hall, University of Delaware, Newark, DE 19716, USA*

Abstract

1,25-Dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) operates through pharmacologically distinct nuclear receptor-mediated and plasma membrane-initiated mechanisms. The nuclear receptor is well described, but the membrane receptor identity remains unproven. A 66 kDa protein from chick intestinal basolateral membranes was isolated previously and identified as a candidate receptor (now termed 1,25D_3-MARRS). A chicken cDNA library was screened for clones encoding the N-terminal sequence of 1,25D_3-MARRS. An exact match was found with an insert containing an open coding region for the full-length candidate 1,25D_3-MARRS protein. Analysis reveals a 5′ untranslated region, a precursor translation product with methionine start site, a signal peptide and a translation product of 505 amino acids prior to translation termination site. Prosite analysis predicts potential sites for phosphorylation by casein kinase II cAMP-dependent kinase, protein kinase C, and tyrosine kinase and an N-myristoylation site with high probability of occurrence. Additionally, two conserved domains capable of interacting with Rel homology domains (RHD) are present. Oligonucleotide primers sets designed to amplify unique regions of the sequence produced amplimers of the predicted size from both chicken and rat intestinal cells. Transcription-translation produced a protein that was recognized in Western blot analysis by Ab099, a polyclonal antibody recognizing the N-terminus of the 66 kDa MARRS protein.

Keywords: 1,25D_3-MARRS; Rapid responses; 1,25-Dihydroxyvitamin D_3; Intestine

1. Introduction

If there is any lesson to have been learned from the world of transcriptionally active nuclear steroid hormone receptor superfamily members it is that proteins seldom function alone. Rather, they function in supramolecular protein complexes that regulate and coordinate a variety of complex cellular functions. The Vitamin D metabolite, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), functions through both nuclear receptor-mediated and plasma membrane-initiated mechanisms that are pharmacologically distinct and part of separate higher order protein complexes. The nuclear receptor is well known and the ligand binding domain (LBD) recently has been crystallized in complex with hormone [1]. Fine details of the manner in which it interacts with its hormone ligand are emerging, and turn out to be quite different from the manner in which 25-hydroxyvitamin D_3 interacts with the serum transport protein known as Vitamin D binding protein (DBP) [2]. The identity of a membrane receptor for 1,25$(OH)_2D_3$ remains unproven although the existence of a membrane complex responsive to 1,25$(OH)_2D_3$ is a certainty [3]. A 66 kDa protein from chick intestinal basolateral membranes was isolated by Nemere and colleagues [4], and identified as a candidate receptor (now termed 1,25D_3-MARRS for 1,25D_3-membrane-associated rapid response to steroid). This brief report describes recent progress in the isolation, cloning and characterization of the candidate receptor, 1,25D_3-MARRS protein, including a demonstration that the mRNA and protein are present in mammalian cells known to demonstrate rapid responses to 1,25$(OH)_2D_3$ [5].

2. Materials and methods

2.1. Cells and tissues

IEC-6 and IEC-18 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and cul-

☆ Presented at the 12th Workshop on Vitamin D (Maastricht, The Netherlands, 6–10 July 2003).

* Corresponding author. Tel.: +1-302-831-2277; fax: +1-302-831-2281.
E-mail address: farachca@udel.edu (M.C. Farach-Carson).
[1] Present address: GlaxoSmithKline, King of Prussia, PA, USA.

0960-0760/$ – see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2004.03.031

tured as stocks in DMEM supplemented with 10% fetal calf serum. Chick basolateral membranes were prepared from Vitamin D-replete white leghorn cockerels as described previously and used as starting material in the isolation of 1,25D_3-MARRS protein or for Western blotting [6].

2.2. Identification of a potential clone for 1,25D_3 MARRS

The 66 kDa 1,25D_3-MARRS protein was isolated as described previously [4]. Back translation to create a degenerate nucleotide sequence was performed using known chicken codon bias and the 20 amino acids of N-terminal sequence available from Edman degradation of the purified 66 kDa protein. A chicken sequence database available through the chicken est project (http://www.chickest.udel.edu) maintained at the Delaware Biotechnology Institute (Newark, DE) was screened for clones encoding the N-terminal sequence of the 66 kDa protein [4]. Exactly one perfect match was found within a stimulated T-lymphocyte library deposited by Dr. Joan Burnside (University of Delaware) that was part of a full-length sequence (start codon through termination sequence) exactly matching the predicted sequence near the 5′ end. The clone was made available to us in BlueScript™ as a stab culture and cultured using standard techniques.

2.3. Isolation and sequencing of plasmid

The QIAprep Maxiprep Spin Protocol from Qiagen (Valencia, CA) was used to recover the plasmid from overnight cultures expanded from ten individual 1,25D_3-MARRS-encoding colonies. Plasmid DNA from each of these 10 individually isolated preparations was sequenced bidirectionally at a DNA sequencing core facility maintained at the Allen Laboratory (University of Delaware). A consensus DNA sequence was generated that had no evident gaps or errors and appeared to represent a full-length 1,25D_3-MARRS sequence.

2.4. Bioinformatic analysis

The full-length 1,25D_3-MARRS protein sequence was analyzed using the Pfam (http://www.sanger.ac.uk/cgi-bin/Pfam/) and ScanProsite (http://us.expasy.org/cgi-bin/scanprosite/) programs to identify structural domains and potential post-translational modification sites. Various alignment programs were also used to compare the sequence to the nuclear receptor for 1,25$(OH)_2D_3$ (nVDR) and to the VDBP.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

0.5 μg of total RNA from chicken intestinal epithelial cells or IEC6/IEC18 cells was reverse transcribed using BD Biosciences Clontech (Palo Alto, CA) Advantage RT-for-PCR kit. PCR was performed using HotStar™ Taq polymerase from Qiagen and primer sets that were developed based on the sequence obtained for the 1,25D_3-MARRS clone. After a 15 min incubation at 95 °C, the cycling conditions were 94 °C for 60 s, annealing at 58 °C for 60 s, and extension at 72 °C for 60 s for 35 cycles. PCR products were visualized on a 1.2% agarose gel containing ethidium bromide. The plasmid containing the 1,25D_3-MARRS encoding insert served as a positive control.

2.6. In vitro transcription and translation

Plasmid containing 1,25D3-MARRS encoding insert was amplified with primers specifically flanking the coding region. This PCR product was ligated into the TA (Invitrogen, Carlsbad, CA) pCRII vector using topoisomerase. *Escherichia coli* were transformed with plasmid and positive clones picked by blue/white selection. Transformed *E. coli* were grown overnight and plasmid DNA isolated by the Qiagen maxiprep method. Orientation of the insert in the vector was determined by restriction digestion with EcoRI and XhoI. Ten microgram of uncut plasmid was linearized by restriction digest at the 3′ end of the open coding region. The linearized plasmid was purified from undigested sequence by low- melt agarose gel electrophoresis. The band was excised from the gel and linearized DNA was purifed from the agarose gel using the Promega (Madison, WI) Wizard DNA prep system. Eluted DNA was transferred to an RNAse free tube, precipitated overnight at −80 °C, rinsed in RNase Free 70% (v/v) EtOH, and resuspended in DEPC-treated water. RNA was transcribed from 2.5 μg of linearized plasmid using T7 polymerase and the Ambion, Inc. (Austin, TX) superscript kit. After transcription for 1 h, DNA was digested with RNAse Free DNAse I. Transcribed RNA was purified by phenol:chloroform:isoamyl extraction, and subsequently treated as described for eluted DNA above. Translation reaction was performed with and without microsomal membranes with the rabbit reticulolysate reaction kit. Typically 2.5–3.0 μg of RNA were used per reaction. All DNA and RNA were quantified by spectrophotometry.

2.7. Western blotting

Reaction products or protein lysates were separated by SDS-PAGE, then transferred to PVDF or nitrocellulose membranes and used for Western blotting with Ab099 as described [4,6].

3. Results

3.1. 1,25D_3-MARRS clone

A sequenced chicken cDNA library made from stimulated T-lymphocytes was screened along with many others in the

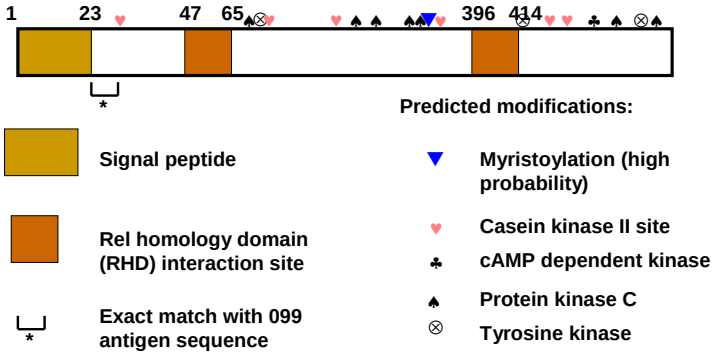

Fig. 1. Schematic diagram of the predicted protein encoded by the full-length 1,25D_3-MARRS clone. The sites for predicted post-translational modifications are indicated with the symbols described in the figure.

database for clones encoding the N-terminal sequence of the 66 kDa protein. An exact match was found with an insert containing an open coding region for the full-length candidate 1,25D_3-MARRS protein. Analysis reveals approximately 50 nucleotides of 5′ untranslated region, a precursor translation product with methionine start site, a 23 amino acid signal peptide and a translation product of 505 amino acids prior to the translation termination site. Prosite analysis of the protein predicts 6 potential casein kinase II phosphorylation sites, 1 cAMP-dependent kinase phosphorylation site, seven protein kinase C phosphorylation sites, three tyrosine kinase phosphorylation sites and an N-myristoylation site with high probability of occurrence. Of great interest, two exactly conserved domains capable of interacting with Rel homology domains (RHD) are also present. A diagram of the predicted 1,25D_3-MARRS clone is shown in Fig. 1. Analysis of the sequence using the NCBI BLAST program revealed a complete absence of similarity to either the nVDR or DBP.

3.2. 1,25D_3-MARRS clone encodes the same protein recognized by Ab099

Oligonucleotide primers based upon the sequences we obtained from the library clone were designed to amplify unique regions downstream from the original matched sequence. Each primer pair produced amplimers of the predicted size from both chicken (not shown) and rat intestinal cell lines IEC-6 and IEC-18 mRNA (Fig. 2), indicating that the clone encodes the same protein recognized by Ab099. In all cases, the sample amplimer products were the same size as those we obtained when amplifying off of the cDNA plasmid indicating that no intronic DNA was present in the samples. Further evidence supporting the belief that our 1,25D_3-MARRS clone encoded the Ab 099 protein was obtained by direct in vitro transcription and translation from the plasmid, followed by Western blotting with Ab099, a polyclonal antibody recognizing the N-terminus of the 66 kDa protein in both chicken and rat. As shown in Fig. 3A, an immunoreactive band of the predicted molecular

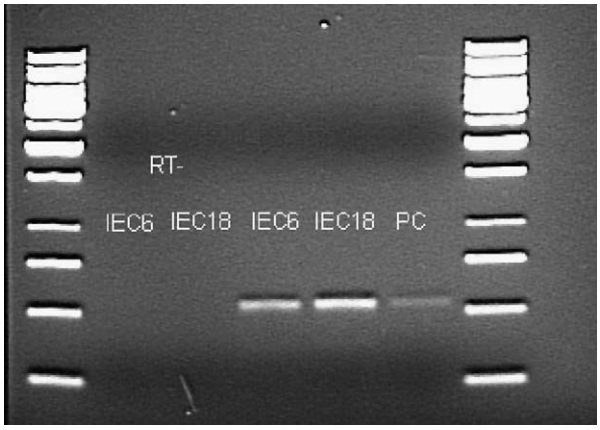

Fig. 2. RT PCR detection of 1,25D_3-MARRS transcripts in two rat intestinal cell lines. Conditions for amplification using 1,25-MARRS specific primers designed from the deduced oligonucleotide sequence are described in the text. The first and last lanes contain size standards, lanes 2 and 3 represent negative controls without reverse transcriptase (no RT), lanes 4 and 5 represent 1,25-MARRS sequence derived amplimers from IEC-6 and IEC-18 cells, and lane 6 a positive control in which the product was amplified directly from the plasmid containing the full-length chicken clone.

weight was present in the translation lysate when full-length MARRS was present (lane 1). In some cases, translation lysates yielded only short truncated immunoreactive protein products of low molecular weight (not shown). This problem is likely to be related to the difficulty of translating chicken mRNAs in mammalian reticulocyte lysate systems, where unusual codon bias reduces efficiency of tRNA loading.

4. Discussion

Together, these data provide strong evidence that the protein encoded by the clone that we identified and sequenced is equivalent to the native protein isolated from chicken basolateral membranes. We previously speculated that the 1,25D_3-MARRS ligand binding domain may have sequence similarity to the nVDR, since both molecules manifested an

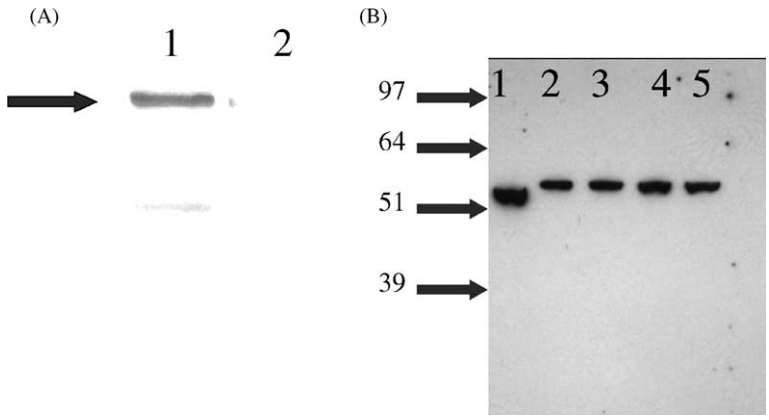

Fig. 3. Western immunodetection of 1,25D_3-MARRS protein. (A) In vitro translation products. Lane 1 shows the detection of the full-length 1,25-MARRS protein produced in vitro and detected with Ab 099 as described in the text. Lane 2 is a negative control lacking full-length product. (B) Lane 1 shows the BLM from chick intestine. Lanes 2 and 3 contain duplicate protein extracts from IEC-6 cells and lanes 4 and 5 show immunoreactive bands from duplicate lanes loaded with IEC-18 cell protein extract. Equal amounts of protein were loaded in lanes 2-5. Arrows denote positions of molecular weight standards.

equivalent affinity for 1,25$(OH)_2D_3$ [6]. However, the findings reported here clearly indicate that the receptor proteins are different molecular entities. 1,25D_3-MARRS, unlike the nVDR has a signal peptide and hence enters the secretory route in the endoplasmic reticulum and Golgi, completely consistent with the immunohistochemical localization reported previously [6]. Although the mature protein is predicted to lack a transmembrane domain, structural analysis indicates that the protein is highly likely to be myristoylated at the site shown in Fig. 1, thus providing an explanation for the association of 1,25D_3-MARRS with biological membranes [6]. The 1,25D_3-MARRS protein also is predicted to possess multiple potential sites for post-translational modification including potential casein kinase II phosphorylation sites, a cAMP-dependent kinase phosphorylation site, protein kinase C phosphorylation sites, and sites for tyrosine kinase phosphorylation. The presence of these conserved consensus sequences primarily in the C-terminal half of the molecule suggests that the activity of the 1,25D_3-MARRS protein can be regulated by the action of cellular signal transduction cascades that have been associated with rapid actions of 1,25$(OH)_2D_3$ [7]. Of particular interest are the sites for the protein kinases A and C, both of which are capable of modifying the activity of Ca^{2+} channels and pumps that modulate plasma membrane Ca^{2+} permeability [8–10].

The small difference in the size of the 1,25D_3-MARRS proteins obtained from chicken BLM or rat IEC cells can be explained either by small sequence differences or by differences in post-translational modification. There are no predicted glycosylation sites in the 1,25D_3-MARRS protein, even though the protein enters the secretory route, so these differences are highly unlikely to be owed to glycosylation. Studies are ongoing to characterize the protein modifications further. During the course of the transcription/translation studies, we frequently observed (not shown) the presence of short apparently truncated N-terminal immunoreactive products, although we were able to obtain the full-length product shown in Fig. 3. We believe that these short in vitro translation products is explained by differences in codon usage between avian and mammalian species, and limitations in tRNA pools needed to translate chicken proteins in mammalian translation competent lysates. We plan to circumvent this problem by using the mammalian 1,25D_3-MARRS homolog for future expression studies, and are creating and characterizing these expression constructs in our laboratories at the present time.

Our immediate future efforts will be directed toward evaluating whether the 1,25D_3-MARRS protein functions as part of an active *complex* involved in transducing the rapid responses to 1,25$(OH)_2D_3$ that have been increasingly recognized [11,12]. Finally, the Rel domains have intriguing implications for the ligand-dependent translocation of 1,25D_3-MARRS protein [13].

Acknowledgements

This work was supported by a grant from the NIH NIDCR RO1 DE12641 (to MCF-C), USDA/CSREES (IN), and NIH NIAMS S11 AR48554 (to SES). The authors thank Ms. Sharron Kingston for expert assistance with the manuscript.

References

[1] N. Rochel, J.M. Wurtz, A. Mitschler, B. Klaholz, D. Moras, The crystal structure of the nuclear receptor for vitamin D bound to its natural ligand, Mol. Cell 5 (1) (2000) 173–179.

[2] M.T. Mizwicki, A.W. Norman, Two key proteins of the vitamin D endocrine system come into crystal clear focus: comparison of the X-ray structures of the nuclear receptor for 1(α, 25$(OH)_2$ vitamin D_3, the plasma vitamin D binding protein and their ligands, J. Bone Miner. Res. 18 (5) (2003) 795–806.

[3] M.C. Farach-Carson, I. Nemere, Membrane receptors for vitamin D steroid hormones: potential new drug targets, Curr. Drug Targets 4 (1) (2003) 67–76.
[4] I. Nemere, M.C. Dormanen, M.W. Hammond, W.H. Okamura, A.W. Norman, Identification of a specific binding protein for 1,25-dihydroxyvitamin D_3 in basal lateral membranes of chick intestinal epithelium and relationship to transcaltachia, J. Biol. Chem. 269 (1994) 23750–23756.
[5] H.J. Armbrecht, M.A. Boltz, T.L. Hodam, V.B. Kumar, Differential responsiveness of intestinal epithelial cells to 1,25-dihydroxyvitamin D_3—role of protein kinase C, J. Endocrinol. 169 (1) (2001) 145–151.
[6] I. Nemere, R. Ray, W. McManus, Immunochemical studies on the putative plasmalemmal receptor for $1,25(OH)_2D_3$. I. Chick intestine, Am. J. Physiol. 278 (2000) E1104–E1114.
[7] B.D. Boyan, V.L. Sylvia, D.D. Dean, Z. Schwartz, Membrane mediated signaling mechanisms are used differently by metabolites of vitamin D_3 in musculoskeletal cells, Steroids 67 (6) (2002) 421–427.
[8] P.C. Gray, J.D. Scott, W.A. Catterall, Regulation of ion channels by cAMP dependent protein kinase and A-kinase anchoring proteins, Curr. Opin. Neurobiol. 8 (3) (1998) 330–334.
[9] J.M. Caffrey, M.C. Farach-Carson, Vitamin D_3 metabolites modulate dihydropyridine-sensitive calcium currents in clonal rat osteosarcoma cells, J. Biol. Chem. 264 (34) (1989) 20265–20274.
[10] T.J. Kamp, J.W. Hell, Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C, Circ. Res. 87 (12) (2000) 1095–1102.
[11] A.W. Norman, J.E. Bishop, C.M. Bula, C.J. Olivera, M.T. Mizwicki, L.P. Zanello, H. Ishida, W.H. Okamura, Molecular tools for study of genomic and rapid signal transduction responses initiated by $1(\alpha, 25(OH)_2$-vitaminD_3, Steroids 67 (6) (2002) 457–466.
[12] R.J. Pietras, I. Nemere, C.M. Szego, Steroid hormone receptors in target cell membranes, Endocrine 14 (3) (2001) 417–427.
[13] B. Tian, A.R. Brasier, Identification of a nuclear factor kappa B-dependent gene network, Recent Prog. Horm. Res. 58 (2003) 95–130.