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Recombinant Expression, Isotope Labeling and Purification of the Vitamin D Receptor Binding Peptide

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The vitamin D receptor binding peptide, VDRBP, was overexpressed as a fused form with the ubiquitin molecule in Rosetta(DE3)pLysS, a protein production strain of *Escherichia coli* harboring an induction controller plasmid. The fusion protein was bound to the immobilized metal ions, and the denaturation and renaturation of the fusion protein were performed as a part of the purification procedure. After the elution of the fusion protein, the peptide hormone was released from its fusion partner by using yeast ubiquitin hydrolase (YUH), and subsequently purified by reverse phase chromatography. The purity of the resulting peptide fragment was checked by MALDI-TOF mass and NMR spectroscopy. The final yields of the target peptide were around 5 and 2 mg per liter of LB and minimal media, respectively. The recombinant expression and purification of this peptide will enable structural and functional studies using multidimensional NMR spectroscopy and X-ray crystallography.

Key Words : Vitamin D receptor, Recombinant peptide, NMR

Introduction

Vitamin D is a fat-soluble secosteroid. The active form in humans is 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), which is involved in bone metabolism and mineral homeostasis.[1] It also has an effect on the proliferation and differentiation of many cell types, and the modulation of immune system.[2,3] This effect on proliferation makes 1α,25$(OH)_2D_3$ a potent anticancer drug.[4] The hormonal effects of 1α,25$(OH)_2D_3$ are initiated upon biding to the vitamin D receptor (VDR), a member of the nuclear receptor superfamily. After this binding, VDR is activated to recruit the retinoid X receptor (RXR), forming a heterodimer.[5,6] This complex is capable of binding to the vitamin D-responsive element (VDRE) with high affinity and initiating the transcription process of vitamin D target genes.[7]

Many agonists, secosteroidal or non-secosteroidal, were developed to increase the desirable antiproliferative and prodifferentiating effects and to decrease the unwanted calcium mobilization and bone resorption in the therapeutic application.[8-10] In some cases like Paget's disease of bone where excessive bone resorption occurs,[11] the vitamin D signal leads to a disease and has to be dealt with. The VDR antagonists were reported to suppress excessive bone resorption, and they are thus therapeutically applicable. Only a few secosteroidal antagonist families were reported to bind the ligand binding domain of VDR.[12-14] In addition to developing an antagonist that directly binds to VDR, there is an alternative way of controlling the vitamin D signaling pathway: manipulating the interactions between VDR/RXR heterodimer and coactivators, which are known to be essential for the expression of vitamin D responsive genes.[15-17] The coactivators contain LXXLL or LLXXL motifs which are essential for the binding to VDR,[18] and this sequence has been reported to interfere with the binding.[19,20] Mita and coworkers showed that LXXLL peptide mimetics could be used as inhibitors.[17]

We have designed a 13-mer peptide containing an LLXXL motif to study the interaction of the peptide and VDR/RXR complex. The sequence was derived from the fragment (residues 625-637) of the coactivator DRIP 205. This peptide was reported to form a ternary complex with VDR and 1α,25$(OH)_2D_3$.[21] Since VDR can bind LLXXL motif, we tried reversing the amino acid sequence, and found that the reversed sequence still retained the comparable binding activity (W. M. Westler and H. F. DeLuca, unpublished result). To better understand the biochemical activities of the peptide, it is important to understand how they behave structurally in both free and bound states. For structural studies, a large amount of sample is needed, and for NMR spectroscopy, it also needs to be labeled, which requires a recombinant expression of the peptide in a suitable host. Here we present our method of producing and purifying recombinant VDRBP by using the ubiquitin fusion system in Rosetta(DE3)pLysS, utilizing the ability of ubiquitin to refold as a purification tool.

Materials and Methods

Construction of VDRBP Expression Plasmid. The gene coding for the peptide, VDRBP (Vitamin D Receptor Binding Peptide), was synthesized chemically (University of

Wisconsin Biotechnology Center, Madison, WI). The sense strand was 5'- ggt ggt aac gat aaa ctg ctg aac atg ctg atg ccg cat aac aaa tga c -3', and the antisense, 5'- cgc cac cat tgc tat ttg acg act tgt acg act acg gcg tat tgt tta ctg agc t -3'. The amino acid sequence of VDRBP is NDKLL NMLMP HNK (13mer). The two DNA strands were annealed, and inserted into the vector pET-28a/ubiS. This vector was slightly modified from its original version, pET-28a/ubi[22-24] in such a way that the 3'-end of the ubiquitin gene sequence was changed to accommodate SacII restriction site without altering the amino acid sequence. This modification yielded a recombinant peptide without additional amino acid residues at the N-terminus.[25] The resulting plasmid was named pET-28a/ubiS/vdrbp.

Expression and Purification of Ubiquitin-VDRBP fusion Protein from an LB Medium. The pET-28a/ubiS/vdrbp plasmid was brought into the expression host, Rosetta(DE3)-pLysS (Novagen, Madison, WI). A single colony was used to inoculate a 100 mL LB medium supplemented with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol. This culture was grown overnight in a shaking incubator at 37 °C. The fully grown culture was used as an inoculum for a fresh one liter LB medium with the same antibiotics the next morning. The culture was grown at 37 °C, and IPTG was added to a final concentration of 0.5 mM when the optical density at 600 nm reached 1.0. The culture was harvested 3 hours later, and the cells were resuspended in 30 mL of 10 mM TrisHCl pH 8.0. The cells were lysed by 'freeze-and-thaw' and the DNA was fragmented by sonication. The soluble fraction was retained after centrifugation at 15,000 rpm for 20 min, and loaded onto HiTrap Chelating HP column (5 mL) charged with Ni^{2+} ions (GE Healthcare, Piscataway, NJ, USA). The column was washed first with 20 mL of Buffer A (10 mM sodium phosphate buffer pH 7.4, 10 mM imidazole, 300 mM NaCl), then 10 mL of 20% ethanol. The proteins that remained in the column were denatured by applying 10 mL of Buffer A containing 8 M urea. On-column refolding was performed by applying 10 mL aliquots in which the urea concentration was reduced stepwise to 6, 4, 2, 1, and 0 M. The column was finally washed with 10 mL Buffer A, and the bound fraction was eluted with 10 mL of Buffer B (10 mM sodium phosphate buffer pH 7.4, 400 mM imidazole, 300 mM NaCl). The ultrafiltration was done both to concentrate the solution and to exchange the buffer to 10 mM sodium phosphate buffer pH 7.4 containing 1 M urea. The amount of protein in the pooled fractions was measured by using the Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Expression and Purification of Ubiquitin-VDRBP Fusion Protein from a Minimal Medium. Rosetta(DE3)pLysS containing the pET-28a/ubiS/vdrbp was grown at 37 °C in a 5 ml LB medium inoculated from a single colony. 1 mL of the fully grown culture was used as an inoculum for a 100 mL of the minimal medium and grown overnight at 37 °C. The fully grown culture was used in turn as an inoculum for a 0.9 liter minimal medium, and the culture was grown at 37 °C. For uniform [^{15}N]-labeling, 1 g of $^{15}NH_4Cl$ per liter culture was provided as a sole nitrogen source. The production and purification steps were the same as the previous section.

Purification of VDRBP. To the ubiquitin-VDRBP fusion protein, β-mercaptoethanol and YUH were added to the final concentrations of 1 mM and 0.1 mg/mL, respectively. The mixture was incubated at 37 °C overnight. The reaction mixture was directly loaded onto a Resource RPC column (GE Healthcare, Piscataway, NJ, USA), and an acetonitrile gradient of 20 to 60% was applied using the HP1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The VDRBP fraction was pooled and lyophilized. The final product was checked by MALDI-TOF. The mass of the peptide was measured directly after lyophilization.

NMR Experiments. The NMR sample contained 1 mM [^{15}N]-VDRBP in 10 mM sodium acetate buffer pH 3.2 and 10% D_2O. The ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) spectrum was collected at 25 °C on a Varian 800 MHz spectrometer. The raw data contained 2048 and 256 complex points in t_2 and of t_1, respectively. The data was processed using the NMRPIPE software package.[26] The final spectrum contained 1024 and 256 real points in t_2 and of t_1, respectively.

Results and Discussion

Construction of Expression Plasmids. The gene coding for ubiquitin-VDRBP was inserted into $(His)_6$-Tag containing vectors to facilitate the purification of the desired proteins. The ubiquitin fusion system was chosen according to the work done by Moon *et al.*.[22] Another reason it was used was to facilitate extensive column washing with the denaturant at high concentration so that non-specific binding proteins are washed away.

Expression and Purification of Ubiquitin-VDRBP Fusion Protein. The expressed proteins appeared as a single band whose size corresponded to ubiquitin/VDRBP fusion protein as shown in lane 8 of Figure 1. The purity of the fusion protein was examined by SDS-PAGE as shown in Figure 1. The final yield of the fusion protein was around 52

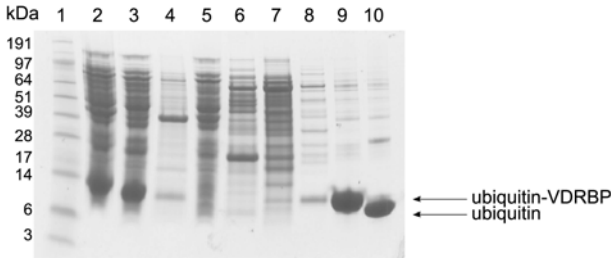

Figure 1. Purification of VDRBP produced from 1 liter LB medium as monitored by 12% SDS-PAGE. Lane 1, size marker; lane 2, whole cell lysate; lanes 3 and 4, supernatant and pellet of cell lysate, respectively; lane 5, flowthrough fraction of supernatant from HiTrap Chelating HP column; lane 6, fraction that was washed out by 20% ethanol; lane 7, fraction that was washed out by Buffer A containing 8M urea; lane 8, fraction bound to the HiTrap column; lane 9, sample of lane 8 after YUH cleavage reaction.

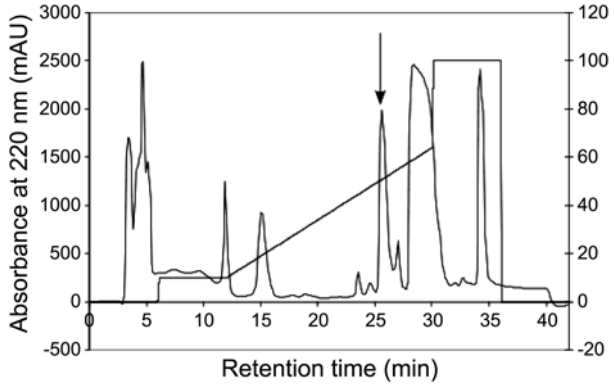

Figure 2. Elution profile from the Resource RPC column on HPLC. The peak corresponding to VDRBP is marked with an arrow. The acetonitrile gradient is also shown.

or 35 mg per liter of LB or minimal medium, respectively.

Purification of VDRBP Peptide. The VDRBP peptide was efficiently clipped off of ubiquitin by YUH. Compared to widely used proteases such as thrombin and TEV protease, a very small amount of YUH (50 μg) was enough to cleave 10-20 mg of the ubiquitin fusion protein. Following the cleavage, the entire reaction mixture was loaded onto a Resource RPC column in 1 mL aliquots on HP1100 HPLC system. The volume of the cleavage reaction mixture was 10 mL, and the reverse phase chromatography was repeated 10 times. The peptide was eluted around at 35% acetonitrile, and all the VDRBP-containing fractions were pooled and lyophilized (Fig. 2). The final yield of VDRBP was around 5 or 2 mg per liter of LB or minimal medium, respectively. MALDI-TOF was used to verify the purified peptide (Fig. 3). MALDI-TOF showed a major peak at 1567.77 Da, which is in good agreement with the theoretical molecular weight of 1567.8 Da.

HSQC Spectrum of ^{15}N-labeled VDRBP Peptide. The HSQC spectrum showed 17 strong and sharp signals with uniform intensity (Fig. 4). Considering the theoretical number of resonances, there should be 11 signals from the backbone, 6 from the sidechain amide groups. The 3 pairs of signals at the upper right corner of the spectrum were characteristic resonances of the sidechain amide groups of Asn or Gln. There are 3 Asn in VDRBP, so these peaks could easily be

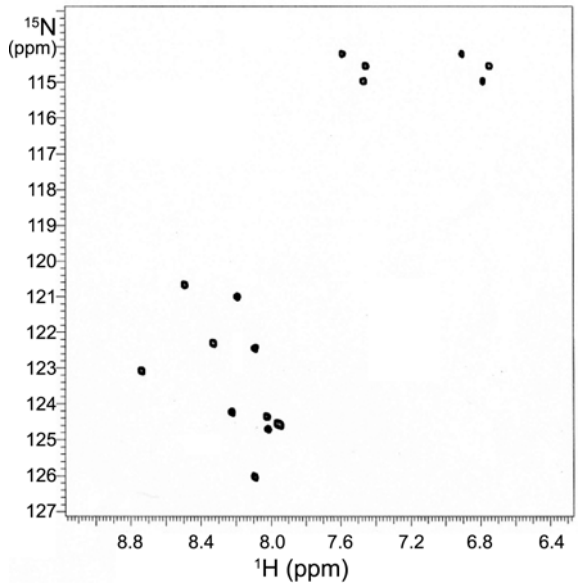

Figure 4. [^{1}H-^{15}N] HSQC spectrum of VDRBP. The NMR sample contained 1 mM [^{15}N]-VDRBP in 10 mM sodium acetate buffer pH 3.0 and 10% D_2O. The spectrum was collected at 25 °C on a Varian Inova 800 MHz spectrometer.

assigned to the sidechain amide groups of Asn-1, 6, or 12; this finding provided further assurance of the purified VDRBP.

Conclusion

The vitamin D receptor binding peptide, VDRBP was recombinantly expressed and purified successfully. The ubiquitin fusion system was proven to be successful again for recombinant VDRBP peptide expression. The greatest advantage of the ubiquitin fusion system over others stems from the small size of ubiquitin, which provides a higher net amount of the target peptides and relatively easier refolding in case of the inclusion body formation. The structural study of the ternary complex of this peptide, VDR, and 1α,25(OH)$_2$D$_3$ will tell us about its detailed binding characteristics, which will lead to the development of a controller in the Vitamin D signal transduction pathway. For this purpose, we have prepared a perdeuterated VDR sample

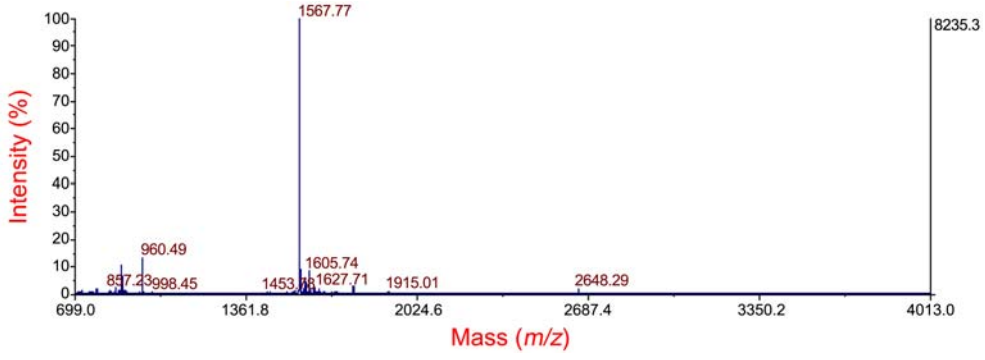

Figure 3. MALDI-TOF spectrum of the VDRBP fraction from HPLC.

so that we can observe the peptide conformation in a bound state and devise a better ligand. The NMR and X-ray structural studies using this method are now in progress.

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