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Development of an Affinity-driven Cross-linker: Isolation of a Vitamin D Receptor Associated Factor

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Abstract—A vitamin D analogue containing an affinity and a photoaffinity probe (affinity-driven cross-linker, **Double Label**) was synthesized. An unknown factor, associated with vitamin D receptor (VDR), was isolated from rat liver nuclear extract using a GST-VDR-ligand-binding domain fusion protein (GST-VDR-LBD), affinity labeled with **Double Label**, and protein–protein cross-linking by photolysis.

Multiple physiological roles of 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), including anti-cancer and immune-regulatory activities, are manifested through a multi-step mechanism that includes highly specific binding of $1,25(OH)_2D_3$ to VDR, heterodimerization of holo-VDR with retinoid X receptor (RXR), binding of VDR-hormone-RXR complex to vitamin D response element (VDRE) in the promoter region of the vitamin D-controlled genes followed by transcription and translation of $1,25(OH)_2D_3$-related proteins.[1–3] It is generally agreed that the ligand-bound VDR-VDRE complex, similar to other nuclear receptor-complexes, recruits many proteins to turn on the transcriptional process; some of them are unique to VDR, and the rest are common among all steriod/retinoid receptor family-members.

During the past few years several nuclear proteins such as TFIIB, SRC-1, GRIP-170, RIP-140, SUG-1, NcoA-62, DRIP, etc., have been isolated by methods that depend critically on the high-affinity non-covalent interaction among proteins such as yeast two-hybrid systems and VDR-affinity chromatography.[4–11] However, it has been amply demonstrated that these factors are common among nuclear steroid/retinoid receptor-family members. We surmise that isolation of factor/factors unique to a nuclear receptor-mediated transcriptional process might require an affinity-driven cross-linking agent that would mimic the natural ligand, and would target and capture a 'protein of interest' while the interacting protein molecules are in close contact.

Several symmetrical and asymmetrical bi-functional cross-linking agents are commerically available.[12] While a symmetrical bi-funcational cross-linking agent (e.g. glutaraldehyde[13]) covalently joins two same or different macromolecules with no selectivity, a hetero bi-functional reagent offers some degree of selectivity. This is achieved by using two dissimilar groups, so that one end attaches to one macromolecule (by alkylation or acylation), while the other end is activated by a 'switch' (often light, and a photoactive probe), and thus, could covalently attach to a different macromolecule. However, these general reagents, lacking affinity for any specific protein (e.g. VDR), cannot be used in a dynamic system like a transcriptional machinery where protein–protein interactions are highly specific and short-lived.

Affinity and photoaffinity labeling reagents, on the other hand, covalently label the binding/catalytically active site of receptors/enzymes by an affinity-driven process,[14] and hence could be suitable for isolating factors in a transcriptional machinery. During the past several years the main focus of our research has been to design, synthesize and develop novel affinity and photoaffinity labeling analogues of $1,25(OH)_2D_3$ and 25-hydroxyvitamin D_3 (25-OH-D_3) to probe the vitamin D sterol-binding pockets of VDR[15–21] and vitamin D-binding protein.[22–30] We have recently demonstrated that 1α,25-dihydroxyvitamin D_3-3β-bromoacetate ($1,25(OH)_2D_3$-3-BE), an affinity analogue of $1,25(OH)_2D_3$,

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specifically labels the $1,25(OH)_2D_3$-binding pocket of nuclear VDR[19–22] and the 'membrane receptor' for $1,25(OH)_2D_3$.[30] Furthermore, we demonstrated that $1,25(OH)_2D_3$-3-BE is transcriptionally active in human skin cells, and acts as a hormone-agonists.[31]

Based on the above observation, we hypothesized that a derivative of $1,25(OH)_2D_3$-3-BE could be designed to carry a photoactive probe at the end of a long tether. Such as a **Double Label**, containing an alkylating group and a photoactive group, would affinity-label the hormone-binding pocket of VDR, and photolysis would generate a highly reactive nitrene intermediate. Thus, when this affinity-labeled VDR is added to a transcriptional assembly in the presence of co-factors (e.g. rat liver nuclear extract, RLNE[32]), photolysis might allow VDR-interacting protein/proteins to be cross-linked to VDR via the nitrene intermediate. Furthermore, choice of VDR (or its ligand-binding domain, LBD) as a fusion partner of glutathione *S*-transferase (GSR-VDT-LBD) might allow the isolation of the VDR-associated factor by affinity chromatography on a Glutathione–Sepharose matrix.[10] Such a possibility is represented schematically in Figure 1.

In the present study we synthesized 19-methyl[6′′-(4-azido-2-nitrophenyl)amino]hexanoyl,25-hydroxy 5E-vitamin D_3-3β-[2′-bromo[acetate (**Double Label**) and its ^{14}C-labeled counterpart (^{14}C-**Double-Label**) by the cyclovitamin D method for introducing functionalities at the C_{19} position of the vitamin D system[33,34] (Fig. 2). The unknown compounds, including the **Double Label**, were characterized by NMR and MS.[35] The radiochemical homogeneity of ^{14}C-**Double Label** was determined by the TLC-analysis of the ^{14}C-**Double Label**, mixed with a sample of the **Double Label** (silica, eluant: 25% ethyl acetate in hexane). Radioactive scanning of the chromatogram showed a single radioactive band coinciding with the same for unlabeled **Double Label**, demonstrating that ^{14}C-**Double Label** was radiochemically homogeneous.

First we carried out affinity labeling studies of VDR-LBD (105-427), expressed as an N-terminal fusion-partner

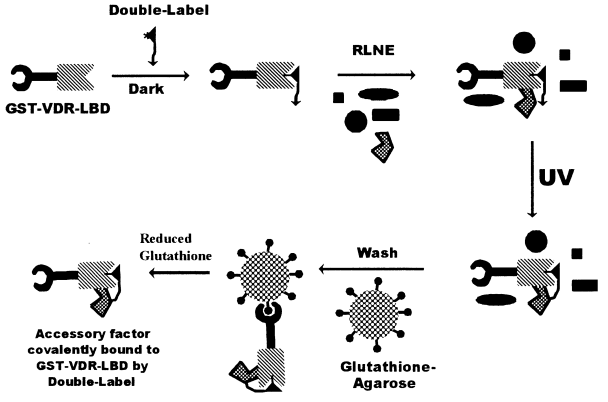

Figure 1. Schematic representation of the utility of a **Double Label** to isolate VDR-assoicated protein/s. GST-VDR-LBD: glutathione *S*-transferase-vitamin D receptor-ligand-binding domain, RLNE: rat liver nuclear extract.

with GST (GST-VDR-LBD)[36] with ^{14}C-$1,25(OH)_2D_3$-3-BE or ^{14}C-**Double-Label** to determine whether ^{14}C-25-OH-D_3-3-BE and ^{14}C-**Double-Label**, without the 1-OH group of ^{14}C-$1,25(OH)_2D_3$-3-BE, are capable of specifically labeling the protein, Incubation of GST-VDR-LBD with ^{14}C-$1,25(OH)_2D_3$-3-BE or ^{14}C-25-OH-D_3-3-BE or ^{14}C-**Double Label** covalently labeled the protein (lane 1 in A, B and C, Fig. 3); and in each case, labeling was significantly reduced in the presence of an excess of $1,25(OH)_2D_3$ (Lane 2 in A, B and C, Fig. 3) demonstrating that ^{14}C-**Double Label**, similar to ^{14}C-$1,25(OH)_2D_3$-3-BE and ^{14}C-25-OH-D_3-3-BE, is capable of covalently modifying the $1,25(OH)_2D_3$-binding pocket of GST-VDR-LBD. These results suggested that the **Double Label** can act as a ligand-mimic for VDR. Additionally, since ligand-binding by VDR is a prerequisite for VDR-mediated transcription, these results also suggested that GST-VDR-LBD, affinity labeled with the **Double Label**, might be able to recruit ingredients for VDR-mediated transcription.

Rat liver nuclear extract (RLNE) is a well-known source of nuclear accessory factors required for saturable hormone-binding and $1,25(OH)_2D_3$-mediated transcriptional process.[32] Thus, we incubated GST-VDR-LBD (20 μg) and RLNE (100 μg) in the presence of protease inhibitor cocktail (2 μg/mL aproptinin 1 μg/mL leupeptin, 1 μg/mL pepstatin and 0.5 mM PMSF) and 3.7 nmol of **Double Label** in a buffer containing 50 mM of Tris–HCl, 150 mM NaCl, 1.5 mM EDTA, and 5 mM DTT, pH 8.3 (TBS-DTT) in the dark for 60 min on ice. At the end of the incubation the samples were exposed to UV radiation (short wave-length, UV-P products, 2.5 cm distance) for 2 min on ice bath. Control experiments were carried out by (a) incubation of GST-VDR-LBD, RLNE and **Double Label** in the presence of 3 μg of $1,25(OH)_2D_3$ and exposure to UV, and (b) incubation of GST-VDR-LBD, RLNE and **Double Label** and no exposure to UV light. In each case, the reaction mixture was subjected to glutathione-Sepharose affinity chromatography to separate GST-VDR-LBD, and associated proteins from the mixture of unrelated proteins in the reaction mixture. The unbound proteins were washed off with TBS-DTT, and the bound proteins were eluated with 20 mM reduced glutathione in TBS-DTT. The eluted proteins were separated on 7% SDS-PAGE and immunoblotted with an anti-VDR antibody.

The immunoblot of the Glutathione-affinity purified protein fraction showed a major shifted band (M_r 95 Kda) along with GST-VDR-LBD band (63 Kda) (Lane 3, Fig. 4). This band was absent with the UV-unexposed sample (Lane 4, Fig. 4) suggesting that 95 Kda band was produced by the photoactivated cross-linking of the **Double Label** to an unknown protein (M_r 32(95–63) Kda) in close physical proximity of the photoactive probe. Since the **Double Label** was anchored to GST-VDR-LBD (63 Kda) via the bromoacetate affinity label this M_r 32 protein must be in close contact with GST-VDR-LBD. Further proof of this phenomenon was provided by the absence of the shifted band when the incubation was carried out in the presence of an excess

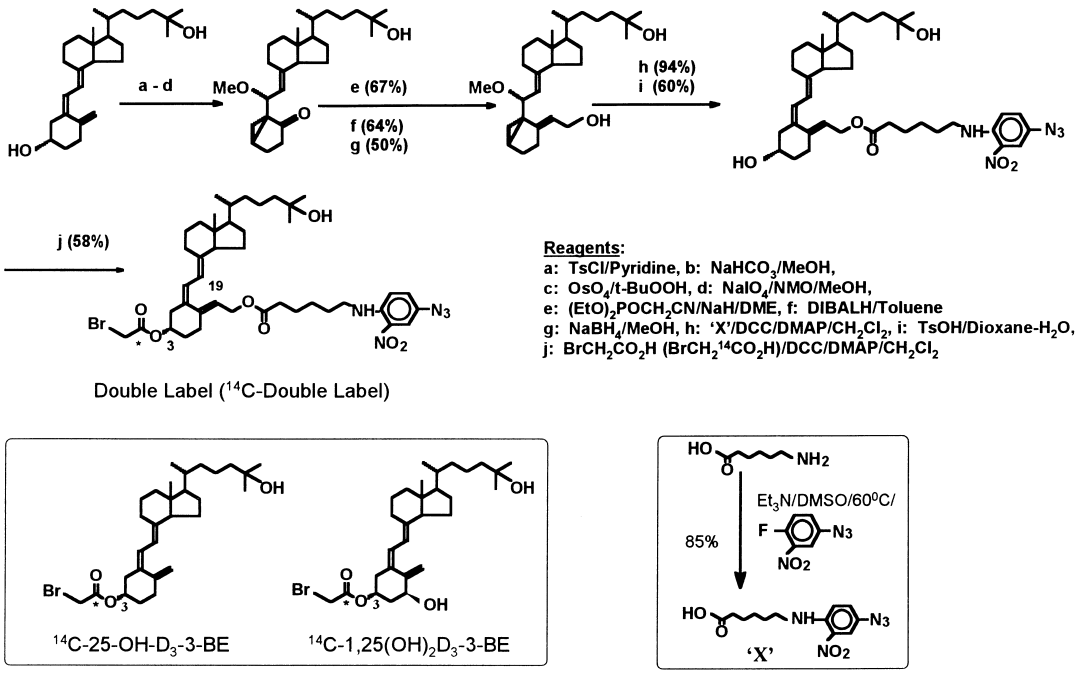

Figure 2. Synthesis of **Double Label** or ^{14}C-**Double Label**; and structures of 25-hydroxyvitamin D_3-3β-bromo[1-^{14}C]acetate (^{14}C-25-OH-D_3-3-BE) and 1α,25-dihydroxyvitamin D_3-3β-bromo([1-^{14}C-1,25$(OH)_2D_3$-3-BE).

of 1,25$(OH)_2D_3$ (Lane 2, Fig. 4) which prevented covalent labeling (anchoring) of GST-VDR-LBD by the **Double Label**.

The identity of the 95 Kda band is yet to be established. RXR is an essential component in the 1,25$(OH)_2D_3$/VDR-mediated transcriptional process. However, the 95 Kda band (in Fig. 4) cannot be GST-GST-VDR-LBD-RXR, because molecular weight of RXR is 50 Kda, and MW of GST-VDR-LBD-RXR should be 113 Kda (63 + 50 Kda). The 95 Kda band also could not be GST-VDR-LBD-dimer, because MW of such a product will be 126 Kda (2×63 Kda). Thus, the 32 Kda protein may represent a 'new' co-factor in the multi-step genomic mechanism involving 1,25$(OH)_2D_3$ and VDR.

In summary, we have designed and synthesized a unique hybrid analogue of 1,25$(OH)_2D_3$ containing an affinity and a photoaffinity probe at different parts of the parent steroid. We have also demonstrated the utility of this analogue in isolating an unknown factor associated with VDR from rat liver nuclear extract. Isolation and identification of this and similar factors, involved in the VDR-mediated transciprtion process, will be critical for the elucidation of the VDR-mediated genomic mechanism; as well as developing 1,25$(OH)_2D_3$-based therapeutic agents for various diseases. It should also be

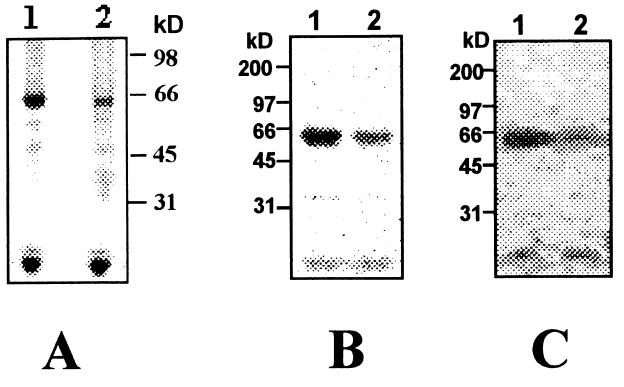

Figure 3. Affinity labeling of GST-VDR-LBD with ^{14}C-1,25$(OH)_2D_3$-3-BE, ^{14}C -25-OH-D_3-3-BE and ^{14}C-**Double Label**. A: Lane 1: GST-VDR-LBD + ^{14}C-1,25$(OH)_2D_3$-3-BE; Lane 2: GST-VDR-LBD + ^{14}C-1,25$(OH)_2D_3$-3-BE + 1,25$(OH)_2D_3$ (excess); B: Lane 1: GST-VDR-LBD + ^{14}C-25-OH-D_3-3-BE, Lane 2: GST-VDR-LBD + ^{14}C-25-OH-D_3-3-BE + 1,25$(OH)_2D_3$ (excess); C: Lane 1: GST-VDR-LBD + ^{14}C-**Double Label**, Lane 2: GST-VDR-LBD + ^{14}C-**Double label** + 1,25$(OH)_2D_3$ (excess).

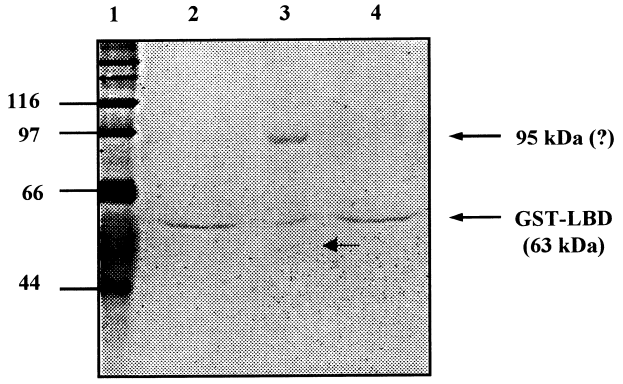

Figure 4. Immunoblot-analysis of samples containing: Lane 1: MW markers; Lane 2: GST-VDR-LBD + RLNE + **Double Label** + an excess of 1,25$(OH)_2D_3$; Lane 3: GST-VDR-LBD + RLNE + **Double Label** + UV; Lane 4: GST-VDR-LBD + RLNE + **Double Label** + no-UV. Positions of other bands are indicated by arrows. The faint band below M_r 63 Kda possibly represents a degradation product.

noted that similar hybrid-analogues of other steroid hormones could be synthesized and used to isolate proteins, associated with corresponding receptors.

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