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Crystallization and Preliminary X-Ray Analysis of a Domain in the Runx2 Transcription Factor That Interacts With the 1α,25 Dihydroxy Vitamin D3 Receptor

Carola Bruna,[1] Gloria Arriagada,[1] Jane B. Lian,[2] Gary S. Stein,[2] Marta Bunster,[1] Jose Martinez-Oyanedel,[1] and Martin Montecino[1]*

[1]Departamento de Bioquimica y Biologia Molecular, Facultad de Ciencias Biologicas, Universidad de Concepcion, Concepcion, Chile
[2]Department of Cell Biology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Abstract The Runx2 transcription factor is a key regulator of osteoblast differentiation. In response to 1α,25 dihydroxy vitamin D3, Runx2 may interact with the 1α,25 dihydroxy vitamin D3 receptor (VDR) in the promoter of target genes, producing a synergic activation of their transcription. Previous studies have suggested that the motifs responsible for the VDR–Runx2 interaction are contained within the 230–361 domain of Runx2. In this work, we confirmed by GST-pull down that $Runx2_{I(209-361)}$ is sufficient to interact with the VDR. To obtain structural information, GST–$Runx2_{I(209-361)}$ protein was overexpressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapor-diffusion method and polyethyleneglycol as a precipitant. The crystals were found to diffract to a maximum resolution of 2.7 Å and a complete data set to a 3.3 Å resolution was collected and analyzed. The crystals belong to the tetragonal system, with a space group *P*4 and unit-cell parameters of a = b = 90.8, and c = 57.2 Å. The presence of a monomer of the recombinant GST–$Runx2_{I(209-361)}$ in the asymmetric unit gives a V_M of 2.7 $Å^3$ Da^{-1} and a solvent content of 54.8%. J. Cell. Biochem. 101: 785–789, 2007.

Key words: 1α,25 dihydroxy vitamin D3 receptor; Runx2 factors; crystallization.

The Runx2 factor is a member of the *runt* homology family of transcription regulators, which is essential for bone formation [Ducy et al., 1997; Choi et al., 2001]. Elimination of the Runx2 gene causes developmental defects in osteogenesis [Komori et al., 1997] and hereditary mutations in this gene are linked to specific ossification defects, as reported in Cleidocranial Dysplasia [Mundlos et al., 1997].

We have recently shown that within the context of the bone-related osteocalcin (OC) gene promoter, there is a tight functional relationship between Runx2 and 1α,25 dihydroxy vitamin D3-dependent pathways [Paredes et al., 2004]. Runx2 and the 1α,25 dihydroxy vitamin D3 receptor (VDR) interact directly in vitro, are components of the same nuclear complexes and colocalize at punctuate foci within the nucleus of osteoblastic cells exposed to 1α,25 dihydroxy vitamin D3 [Paredes et al., 2004]. The protein–protein interaction between Runx2 and VDR requires a domain located C-terminal of the runt homology DNA-binding region, spanning residues 230 to 361. Interestingly, this same domain is also required for interaction of Runx2 with the transcriptional co-activator p300 [Sierra et al., 2003], which can be recruited by Runx2 to bone-related target genes and thus regulate their transcription.

Although the function of the 230–361 domain of Runx2 has been experimentally defined, the

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*Correspondence to: Martin Montecino, Departamento de Bioquimica y Biologia Molecular, Facultad de Ciencias Biologicas, Universidad de Concepcion, Casilla 160-C, Concepcion, Chile. E-mail: mmonteci@udec.cl

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critical residues required for each key protein–protein interaction have not been identified. To this end and to establish the tertiary structure of this regulatory domain, we have cloned the region encoding amino acid residues 209 to 361 of the mouse $Runx2_I$ protein in fusion with the glutathione-S-transferase (GST) gene and produced recombinant GST–$Runx2_{I(209-361)}$ protein in *E.coli*. We report that the 209–361 domain of $Runx2_I$ retains the ability to interact specifically with the VDR and that it forms crystals that diffract up to 2.7 Å.

MATERIALS AND METHODS

Protein Expression and Purification

Generation of the GST–$Runx2_{I(209-361)}$ plasmid, coding for residues 209–361 of mouse $Runx2_I$ (p56) fused to GST, has been described previously [Paredes et al., 2004]. GST–$Runx2_{I(209-361)}$ protein was overexpressed in *E.coli* BL-21(DE3) cells. Cells were grown at 303 K to an OD_{600} of 0.6 in Luria-Bertani medium containing 100 μg/ml ampicillin. Protein expression was induced by 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and cell growth was allowed to continue for 3 h at 298 K. After induction, cells were harvested by centrifugation at 6,000*g* for 10 min at 277 K. The cell pellet was suspended in ice-cold PBS (1.5 mM KH_2PO_4, 8 mM $Na_2HPO_4 \cdot 2H_2O$, 137 mM NaCl, 2.7 mM KCl, pH 7.4) supplemented with a complete protease inhibitor cocktail (Roche Diagnostics Gmbc, Mannheim, Germany) and 1 mM dithiothreitol (DTT). Cells were lysed by sonication with an ultrasonic processor. The crude cell extract was centrifuged at 12,000*g* for 10 min at 277 K and the recombinant protein in the supernatant was purified by glutathione sepharose affinity chromatography (Amersham Biosciences AB, Uppsala, Sweden). The protein content of the eluates was determined as previously reported [Bradford, 1976] and their composition analyzed by dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) [Laemmli, 1970] and Western blot [Towbin and Gordon, 1984] using an anti-GST goat polyclonal antibody (Rockland Immunochemicals, Inc., Gilbertsville, PA) and an anti-Runx2 rabbit polyclonal antibody directed against its C-terminus (M-70, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Two milligrams of total protein from the selected eluate was subjected to a second glutathione sepharose affinity chromatography. The selected eluate was then concentrated and transfered into 100 mM HEPES buffer pH 7.5 using a YM-10 ultrafiltration membrane (Amicon, Bedford, MA). The protein concentration was estimated by measuring the absorbance at 280 nm.

The expression construct pGEX-$Runx2_I$ encoding the wild type mouse $Runx2_I$ protein (p56) was prepared as reported previously [Paredes et al., 2004]. pGEX-$Runx2_I$ was cotransformed with plasmid pREP4 (Qiagen, Inc., Valencia, CA), a multicopy repressor plasmid which ensures high levels of the lactose repressor, in *E.coli* BL-21 (DE3) cells. Expression of GST–$Runx2_I$ was induced by 25 μM IPTG for 1 h at 298 K in cells grown to an OD_{600} of 0.75 at 298 K. The recombinant protein was purified by glutathione sepharose affinity chromatography. Eluates were analyzed by Bradford's, SDS–PAGE and Western blot with an anti-GST goat polyclonal antibody (Rockland Immunochemicals, Inc.).

Cloning of the VDR gene into the pGEX5X3 vector has been described previously [Paredes et al., 2004]. The recombinant protein was overexpressed in *E.coli* BL-21 (DE3) cells grown to an OD_{600} of 0.4 at 310 K. Induction was accomplished by the addition of 200 μM IPTG for 3 h at 300 K. GST–VDR was immobilized in a glutathione sepharose column and the GST tag was removed by an overnight incubation with 1 U Factor Xa at 277 K (New England Biolabs, Beverly). The untagged protein was collected and analyzed by Bradford's, SDS–PAGE and Western blot with an anti-VDR rabbit polyclonal antibody directed against its C-terminal (C-20, Santa Cruz Biotechnologies, Inc., Santa Cruz).

GST-Pull Down Assay. Assays were performed in 25 μl of glutathione sepharose beads (Amersham Biosciences AB). GST or GST fusion protein (1 μg) was incubated in 400 μl of binding buffer (20 mM Tris-Cl pH 8.0, 100 mM KCl, 0.5% NP-40, 10 mM ethylene diamine tetra acetic acid (EDTA), 0.05 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT) for 30 min at 277 K with gentle agitation. Beads were collected by centrifugation at 2,500*g* for 30 s at room temperature and washed with 500 μl of binding buffer for 5 min at 277 K. After collection, beads were incubated with 1.5 μg of purified VDR in a total volume of 500 μl of binding buffer with 0.5% w/v milk for 2 h at 277 K with gentle agitation. Following four washes with 500 μl of

binding buffer for 5 min at 277 K, beads were suspended in 20 μl of loading buffer 2× (100 mM Tris-Cl pH 6.8, 4% (w/v) SDS, 0.15% (v/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT) and incubated for 5 min at 368 K. Samples were fractionated by SDS–PAGE at 10% acrylamide and the presence of VDR and the GST fusion proteins was determined by Western blot with anti-VDR (C-20, Santa Cruz Biotechnologies, Inc.) and anti-GST (Rockland Immunochemicals, Inc.) antibodies, respectively.

Crystallization

Crystals were grown by the hanging-drop vapor-diffusion method at 291 K. Initial screening was performed using the commercial kit Structure Screen 1 MD1-01 (Molecular Dimensions Limited, Cambridgeshire, UK). A volume of 1 μl of precipitating agent from a 500 μl reservoir was added to 1 μl of GST–$Runx2_{I(209-361)}$ protein at 8 mg/ml in 100 mM HEPES pH 7.5. Within 3 days, irregular crystals appeared in 0.1 M Tris HCl pH 8.5, 30% w/v polyethyleneglycol (PEG) 4000, 0.2 M $MgCl_2 \cdot 2H_2O$. To improve the quality of the crystals, slight variations in either the composition of the crystallization solution, the protein concentration, or the protein and crystallization solution ratio were assessed. In addition, streak seeding and microseeding assays were also performed.

X-Ray Diffraction Analysis

To avoid radiation damage, crystals were mounted in a nylon loop and cryo-cooled in liquid nitrogen using mother liquor solution as cryoprotectant. X-ray diffraction data were collected at 100 K with a MAR Research CCD detector at the B03D-MX1 beamline at the National Laboratory of Synchrotron light (LNLS), Campinas, Brazil using 1.438 Å X-rays. The crystal was rotated for a total of 199°, with 1.0° oscillation per frame and crystal-to-detector distance set to 140 mm. The data set was indexed and scaled using the programs MOSFLM [Leslie, 1994] and SCALA from the CCP4 suite [Collaborative Computational Project, Number 4, 1994], respectively.

RESULTS AND DISCUSSION

As described in Materials and Methods, conditions for optimal expression of the GST–$Runx2_{I(209-361)}$ protein in *E.coli* were assessed. This protocol yielded a GST–$Runx2_{I(209-361)}$ protein fraction devoid of significant cleavage products detected by Western blot, with adequate quality for subsequent crystallization studies (Fig. 1). The yield was approximately 15 mg per liter of bacterial culture.

Runx2 is a key regulator for transcription of osseous-specific genes [Lian et al., 2004]. Treatment of osteoblastic cells with 1α,25 dihydroxy vitamin D3 results in stimulation of the expression of bone-specific genes such as osteocalcin (OC). This is in part due to the direct protein–protein interaction between the VDR and the Runx2 factor, while both proteins simultaneously bind to their cognate elements within the OC promoter [Paredes et al., 2004]. As shown in Figure 2, the interaction between Runx2 and VDR in vitro can be demonstrated by GST pull-down analysis, using either purified recombinant full-length GST–$Runx2_I$ or GST–$Runx2_{I(209-361)}$ proteins. Although the VDR–GST–$Runx2_{I(209-361)}$ interaction is slightly weaker than that exhibited by the full-length Runx2 protein (Fig. 2, compare lanes 3 and 4), GST–$Runx2_{I(209-361)}$ retains the ability to precipitate VDR efficiently. This result confirms that the 209–361 domain of Runx2 is sufficient to bind VDR [Paredes et al., 2004] and that the structural components required for this interaction are maintained in the GST–$Runx2_{I(209-361)}$ protein. Therefore, crystallization and structural analyses of the GST–$Runx2_{I(209-361)}$ protein may yield important information to understand the basic molecular components of Runx2–VDR complex formation.

Small irregular crystals appeared after incubation of the GST–$Runx2_{I(209-361)}$ protein for

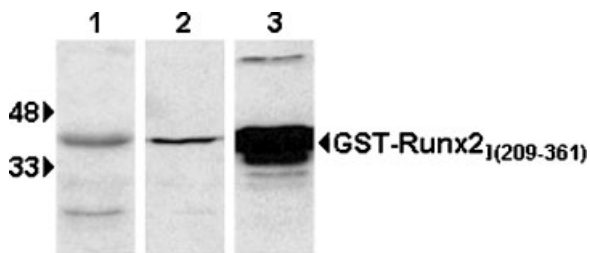

Fig. 1. Expression of $Runx2_{I(209-361)}$ protein in *E.coli*. **Panel 1**: Coomassie-stained SDS–PAGE containing 2 μg of purified recombinant protein. **Panel 2**: Western blot analysis of 0.2 μg of purified $Runx2_{I(209-361)}$ protein using an anti-GST polyclonal antibody. **Panel 3**: Western blot analysis of 0.2 μg of purified $Runx2_{I(209-361)}$ protein using an anti-Runx2 rabbit polyclonal antibody directed against its C-terminus. Migration of molecular weight markers is indicated on the left.

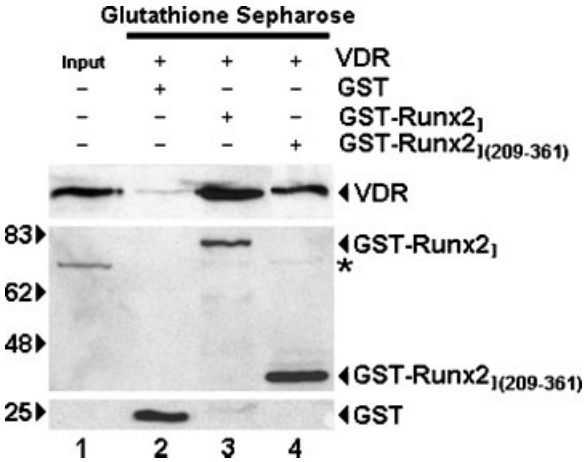

Fig. 2. $Runx2_{I(209-361)}$ interacts with VDR in vitro. Full-length Runx2 and $Runx2_{I(209-361)}$ protein were expressed as GST-tagged fusion proteins and their ability to interact in vitro with recombinant VDR was evaluated by GST pull-down assays. GST or the GST fusion proteins (1.0 μg) bound to 25 μl of glutathione sepharose resin were incubated with VDR (1.5 μg) for 2 h at 277 K. Precipitated proteins were analyzed by Western blotting with specific antibodies against VDR (**upper panel**) or GST (**lower panels**). The combinations of recombinant proteins for each binding assay are indicated at the top. The position of molecular mass markers is indicated on the left of the blots. The asterisk indicates a non-specific recognition.

3 days in a buffer containing 0.1 M Tris HCl pH 8.5, 30% (w/v) PEG 4000, 0.2 M $MgCl_2 \cdot (H_2O)_6$ using the hanging-drop vapor-diffusion method (Fig. 3). In order to generate high quality crystals, $GST-Runx2_{I(209-361)}$ protein was subjected to a second round of glutathione affinity chromatography with no apparent change in the stability of the protein (data not shown). Nevertheless, this step was crucial for a successful crystallization. Protein samples were used immediately for crystallization assays and could not be stored or frozen. No significant improvement in the quality of the crystals was observed after changing the protein concentration or the protein and crystallization solution ratios. Similarly, steak seeding or microseeding assays did not change the results.

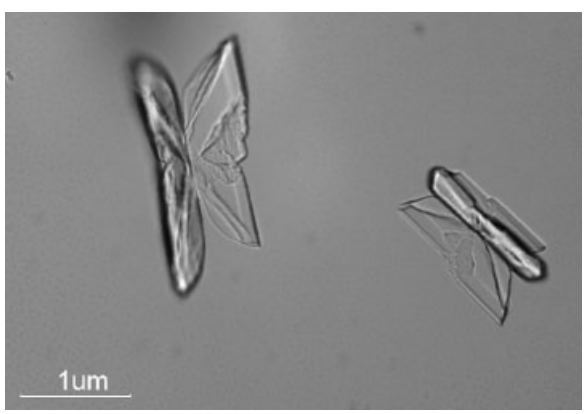

Fig. 3. Crystals of the mouse $GST-Runx2_{I(209-361)}$ protein grown by the hanging-drop method.

TABLE I. Data-Collection Statistics

Values in parentheses are for the highest resolution shell (3.48–3.30 Å)

Space group	*P*4
Unit-cell parameters (Å)	a = b = 90.8, c = 57.2
Resolution range (Å)	27.3–3.3
Rotation per exposure (°)	1.0
Time per image (s)	360
Temperature (K)	100
Measured reflections	57,361 (8425)
Independent reflections	7,180 (1052)
Completeness (%)	99.8 (100.0)
Multiplicity	8.0 (8.0)
Mean I/σ (I)	4.7 (2.0)
R_{merge} (%)	14.8 (36.4)

On the basis of systematic absences and statistical comparison, the crystal was classified into the tetragonal space group *P*4. The data-collection statistics are summarized in Table I. The data are 99.8% complete to 3.3 Å resolution, with a total of 57,361 measured reflections. R_{merge} for the entire data set collected from a single crystal is 14.8%. The highest resolution shell is 100% complete, with a multiplicity of 8.0 and a R_{merge} of 36.4%. Figure 4 shows a representative X-ray diffraction image obtained from a $Runx2_{I(209-361)}$ crystal. The unit-cell parameters refined to a = b = 90.8, and c = 57.2 Å. According to the calculated Matthews coefficient [Matthews, 1968], there is one molecule per asymmetric unit, with a corresponding crystal volume per protein weight of 2.7 $Å^3$/Da and a solvent content of 54.8%. A self-rotation function confirmed the space group selection. In summary, we show that the 209–361 domain present in our purified $GST-Runx2_{I(209-361)}$ protein retains the ability to interact specifically with VDR. This result indicates that this region of Runx2 contains an independent domain that is responsible and sufficient for binding VDR. Therefore, our $GST-Runx2_{I(209-361)}$ protein fraction is suitable for structural analysis to obtain information about the specific motifs involved in the VDR–Runx2 interaction.

Butterfly-shaped crystals of $GST-Runx2_{I(209-361)}$ were formed within 3 days. These crystals exhibited high stability, which facilitated their

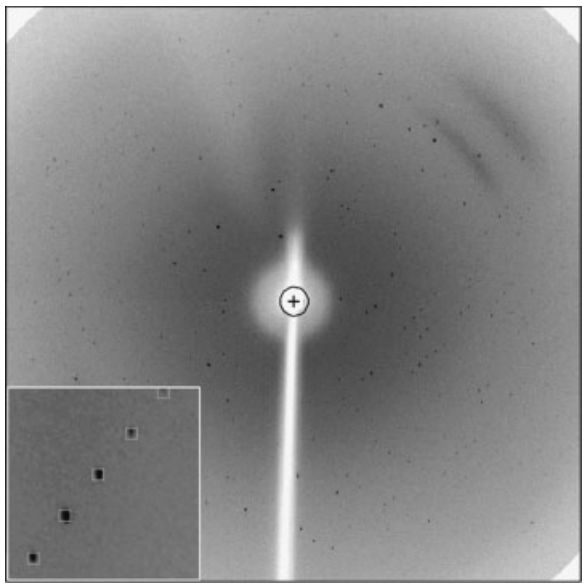

Fig. 4. X-ray diffraction image from the GST–Runx2$_{I(209-361)}$ crystal recorded at 100 K on a MAR Research CCD at the LNLS. The panel at the bottom is an enlarged image that shows the agreement between the experimental diffraction pattern and the predicted reflections from MOSFLM (represented as squares).

transportation to the synchrotron facility and the subsequent analyses. We report the production of Runx2$_{I(209-361)}$ crystals that diffract up to 2.7 Å. Based on the value of the R_{merge} for the highest resolution shell, the selected resolution limit was 3.3 Å. Lowering the resolution did not significantly decrease the overall R_{merge}. Because the tertiary structure of the GST protein is available, the structure of the Runx2$_{I(209-361)}$ fusion protein will be solved by the molecular replacement method [Navaza, 1994].

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