J Hum Genet (2005) 50:679–683
DOI 10.1007/s10038-005-0311-3

SHORT COMMUNICATION

**Cinzia Puppin · Lucia Pellizzari · Dora Fabbro
Federico Fogolari · Gianluca Tell · Alessanda Tessa
Filippo M. Santorelli · Giuseppe Damante**

Functional analysis of a novel *RUNX2* missense mutation found in a family with cleidocranial dysplasia

Received: 20 July 2005 / Accepted: 30 August 2005 / Published online: 22 October 2005

Abstract Mutations of the *RUNX2* gene result in dominantly inherited cleidocranial dysplasia (CCD). *RUNX2* encodes for an osteoblast-specific transcription factor, which recognizes specific DNA sequences by the runt domain. DNA binding is stabilized by the interaction with the protein CBFβ, which induces structural modifications of the runt domain. A novel 574G > A *RUNX2* missense mutation has been found in members of a family clinically diagnosed with CCD. This mutation causes the glycine at position 192 to change to arginine (G192R), in loop 9 of the runt domain. Unlike other residues of loop 9, G192 does not establish DNA contacts. Accordingly, the G192R mutant showed a 50% reduction in binding activity compared to the wild-type runt domain. However, the mutation completely abolished the activating properties of the protein on osteocalcin promoter. Moreover, the G192R mutant exerts a dominant-negative effect when overexpressed. Computer modeling indicated that the G192R mutation perturbs not only loop 9, but also other parts of the runt domain, suggesting impairment of the interaction with CBFβ.

Keywords *RUNX2* · Cleidocranial dysplasia · Transcription factor · DNA binding · Osteoblast · Bone

C. Puppin and L. Pellizzari contributed equally to the work.

C. Puppin · F. Fogolari · G. Tell
Dipartimento di Scienze e Tecnologie Biomediche,
Università di Udine, Piazzale Kolbe 1, 33100 Udine, Italy

L. Pellizzari · D. Fabbro · G. Damante (✉)
Istituto di Genetica, Azienda Policlinico Universitario Udine,
Udine, Italy
E-mail: gdamante@mail.dstb.uniud.it
Tel.: +39-432-494370
Fax: +39-432-494379

A. Tessa · F. M. Santorelli
Dipartimento di Medicina Molecolare,
IRCCS–Bambino Gesù, Roma, Italy

Introduction

Cleidocranial dysplasia (CCD) is an autosomal-dominant inherited skeletal disease with high penetrance and variable expressivity, characterized by hypoplastic/aplastic clavicles, large fontanelles, dental anomalies, and delayed skeletal development (OMIM #119600). It has been established that *CBFA1*/*RUNX2* is the gene responsible for an equivalent phenotype in heterozygous mice and molecular studies have identified several mutations of this gene in patients with a clinical diagnosis of CCD (Mundlos 1999). *RUNX2* encodes for a transcription factor that belongs to the CBFα family, which is characterized by the 128 amino acids long evolutionary conserved, runt domain, and is homologous to the *Drosophila* pair-rule gene *runt*. *RUNX2* controls osteoblast differentiation, specifically through heterodimerization with CBFβ and by binding to specific DNA sequences (Ducy et al 1997). CBFβ stabilizes the *RUNX2*–DNA interaction (Tahirov et al 2001). Several mutations impairing *RUNX2* activity have been identified and characterized in patients with CCD, including both familial and sporadic cases (Quack et al 1999; Otto et al 2002). Most of these mutations are missense mutations in the runt domain that impair both specific DNA binding and promoter transactivation (Lee et al 1997; Yoshida et al 2003).

The aim of this study was the analysis of a novel *RUNX2* missense mutation found in a family with CCD.

Materials and methods

Patients and mutation screening

The family analyzed was initially referred for genetic counseling to the Istituto di Genetica of Policlinico Universitario in Udine (Italy). Informed consent was given for us to obtain blood samples for genetic analysis.

Total DNA was purified from peripheral blood leukocytes of patients, and the mutation screening was

performed on the eight exons (from zero to seven) of the *RUNX2* gene, as previously described (Tessa et al 2003).

Plasmids, mutagenesis, and expression of recombinant proteins

To express the recombinant runt domain we used the pGST-OSF2 plasmid encoding the *RUNX2* cDNA cloned in frame with GST in the pGEX4T3 plasmid (Pharmacia, Piscataway, NJ, USA). For transactivation studies, the following plasmids were used: pCMV-OSF2 encoding the full length mouse *RUNX2* cDNA under the control of CMV promoter in the pCMV5 plasmid; p6OSE2-luc encoding six OSE2 target sequences upstream of the luciferase (luc) reporter gene (Ducy et al. 1999). The mutation G192R was introduced into both eukaryotic and prokaryotic vectors using a Quick-change kit (Stratagene, La Jolla, CA, USA), following the manufacturer's instructions. Recombinant proteins were obtained in a BL21 *Escherichia coli* strain by IPTG induction as previously described (Damante et al 1996).

Gel retardation assay and cell transfection

The oligonucleotide sequences used as probes were as follows. The wild-type, 5′-CGCAGCTCCC*AACCACA* TATCCTCT-3′ (top strand) is derived from the human osteocalcin promoter (−141 to −165) and contains an OSE2 motif (AACCACA). The mutant sequence is 5′-CGCAGCTCCC*AGACACA* TATCCTCT-3′ (top strand) (Ducy et al. 1997). Double-stranded sequences labeled at the 5′ end with ^{32}P were used as probes in gel retardation assays, which were performed as previously described (Damante et al 1996). HeLa cells were cultured in DMEM medium with 10% calf serum. The calcium phosphate coprecipitation method was used for transfections, as described elsewhere (Puppin et al 2003).

Computer simulations of *RUNX2* wild-type and G192R mutant structures

The sequence for *Homo sapiens RUNX2* was obtained from GenBank (Acc. NP_004339.3). The fragment selected for modeling starts at amino acid 97 and ends at amino acid 210. The mutation addressed in the present study occurs at position 178 in this sequence, but we will use the number 192 for consistency with previous studies (Lee et al 1997). This fragment, covering most of the runt domain, was created via homology modeling on the runt domain of the *RUNX1* domain, solved by X-ray crystallography at 1.7 Å resolution (chain A, pdb id. 1ean) (Bäckström et al 2002). Model building for the native form and the G192R mutant was performed using the program Nest (Petrey et al 2003). Model building was straightforward because of the high degree of identity (104/114 residues) and overall similarity (110/114 residues) between the *RUNX1* and *RUNX2* runt domain sequence. The system was prepared and simulated as previously described for β-lactoglobulin (Fogolari et al 2005). In summary, the system was energetically minimized and ions were added in the most stable regions according to the solution of the Poisson–Boltzmann equation, in order to make the system electrostatically neutral. The system (protein and ions) was placed in a box of water of size 82×66×63 Å^3. Water was energetically minimized and 100 ps of molecular dynamics (keeping the protein and ions fixed) were simulated in order to let the water soak the protein. Then the whole system was energetically minimized and equilibrated to 300 K for 100 ps. Finally, 3.6 ns of molecular dynamics were simulated and used for later analysis.

Results and discussion

The family analyzed was composed of a 55-year-old mother and two children: a 27-year-old male and a 33-year-old female. All patients had a clinical diagnosis of CCD and displayed a classic phenotype with aplasia of the clavicles, large fontanelles, short stature, skeletal anomalies, and supernumerary teeth. Aplasia of the clavicles was monolateral in the son. The mother suffered aseptic nontraumatic necrosis of the femoral head at the age of 7. At the age of 55 she was still menstruating. In this subject, at the age of 48, BMD data indicated the occurrence of osteopenia (L2–L4 T and Z scores −2.07 and −1.41, respectively). The mutation, initially found in the mother, was also confirmed in both children. The mutation affects position 574G > A in *RUNX2* exon 2, causing the glycine at position 192 to change into arginine (G192R) in loop 9 of the runt domain, and it has been never described before. Gel retardation experiments, performed with wild-type and mutant recombinant proteins, show that the G192R mutant maintains a DNA binding activity that is about 50% of that of the wild-type protein (Fig. 1). The mutant protein retains DNA binding specificity, as demonstrated by the inability to bind the OSE2 mutant oligonucleotide.

An analysis of the transactivation properties of the G192R mutant was performed via cotransfection assays in HeLa cells, using the *RUNX2*-responsive osteocalcin promoter linked to the luc gene (p6OSE2-luc) as reporter. As shown in Fig. 2a, the G192R mutation abolishes the specific transactivation properties of *RUNX2* on the specific target promoter. In Fig. 2b, we can see that the G192R mutant, when overexpressed, exerts a dominant-negative effect. In fact, an excess of the expression vector for the mutant protein (9 μg versus 1 μg), reduces the transactivating effect exerted by the expression vector of the wild-type protein. This effect is not due to a squelching phenomenon (Gill and Ptashne 1988), as demonstrated by using 10 μg of the wild-type expression vector. A simple explanation for these results is that the mutant protein is able to interact with CBFβ, reducing the possibility of interactions between CBFβ

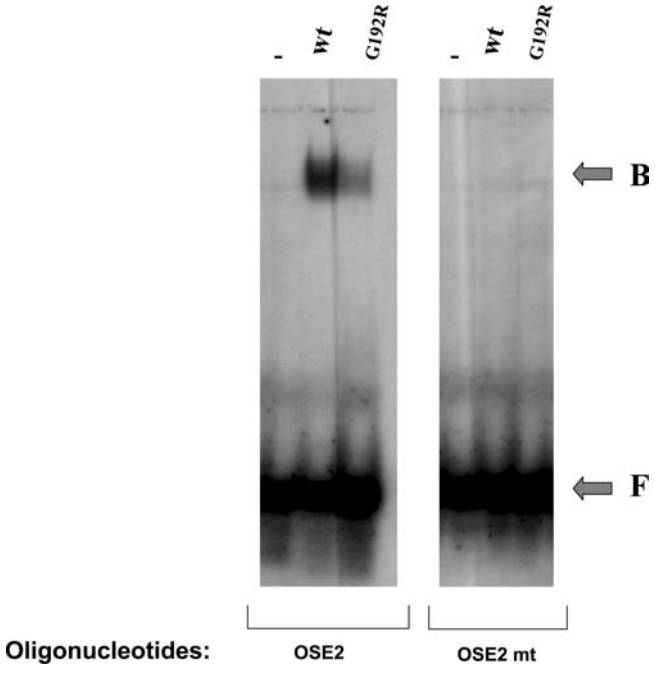


Fig. 1 DNA binding of wild-type and G192R mutant to a target sequence of *RUNX2* (OSE2) and to a mutant form of it (OSE2 mutant). *B* and *F* indicate protein-bound and free DNA, respectively

and the wild-type protein. The interaction between the mutant protein and CBFβ is not productive—the latter is not able to induce any structural modification that contributes to the stabilization of the protein–DNA complex. Accordingly, structural modification of loop 9 of the runt domain occurs upon interaction with CBFβ (Tahirov et al 2001; Bäckström et al 2002). From these data, it cannot be concluded whether the patient's phenotype is influenced by the dominant-negative effect of the mutation. However, it is important to note that the older family patient (the mother), though menstruating, suffers from osteopenia (which has only been reported in a few CCD patients). This finding fits with the observation that transgenic mice expressing a dominant-negative allele of *RUNX2* after birth develop an osteopenic phenotype (Ducy et al 1999).

To support the hypothesis delineated above, we have performed molecular dynamics simulations on the wild-type and G192R mutant uncomplexed protein. As expected, the backbone conformation at position 192 is lost when native glycine is replaced by an arginine. This conformational rearrangement, which takes place during the 100-ps equilibration simulation, is the likely molecular basis for all the observed effects. For further analysis, snapshots were taken at 100 ps intervals along the dynamics. In order to simplify the analysis, snapshots were superimposed pairwise and the structure possessing the smallest average root mean square deviation (RMSD), computed on Cα atoms, among the snapshots, was taken as the most representative conformation of the ensemble. The structures chosen were those at 2.0 ns (average RMSD = 1.13 ± 0.19 Å) and 3.3 ns (average RMSD = 1.20 ± 0.41 Å) for native and mutant simulations, respectively.

The RMSD between the Cα atoms of the two most representative conformations is reported in Fig. 3a. A very large deviation (over 5 Å) is observed at the region encompassing the mutated residue. The conformational change occurring in loop 9 is able to propagate by a network of hydrogen bonds to neighboring loop 5. It is expected that any change in one of these two loops will

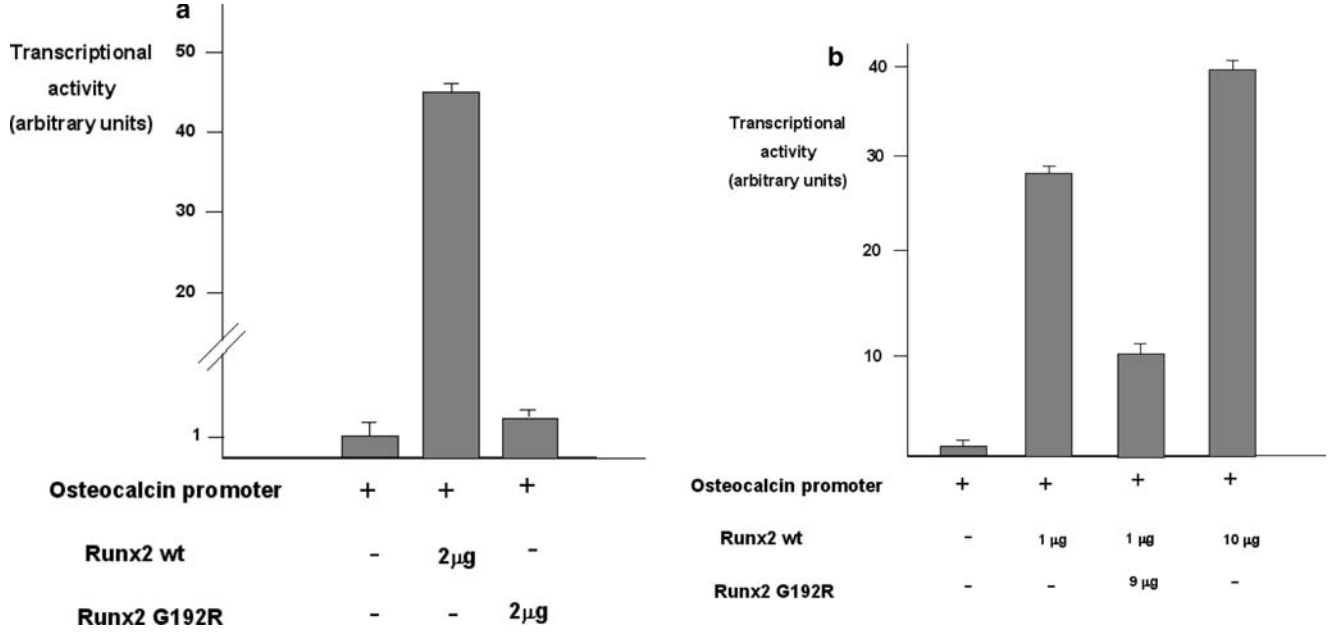
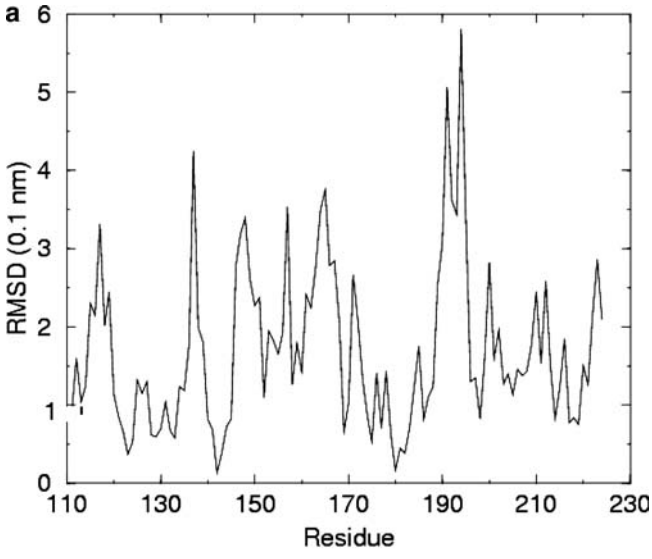


Fig. 2a,b Transcriptional effects of the wild-type *RUNX2* and the G192R mutant on osteocalcin promoter. **a** The G192R mutant is not able to activate the osteocalcin promoter. **b** The G192R mutant exerts a dominant-negative effect on the wild-type protein. In both **a** and **b**, each *bar* indicates the mean value ± SD of three independent transfections



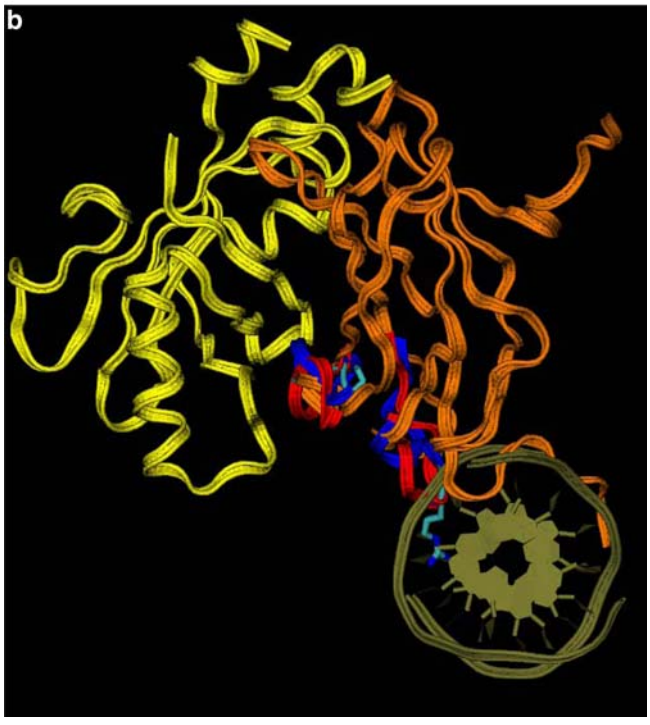


Fig. 3a,b **a** Root mean square deviation (in Å) for Cα atoms after global superposition of the most representative structures for the native form and the G192R mutant of the runt domain. **b** Loops 5 and 9 of the native form (*blue*) and the G192R mutant (*red*) of the runt domain. Residues 192 and 160, important for loop 5 and 9 interactions, are displayed in bond representation. The two most representative structures of the native and mutant ensembles are superimposed on the crystallographic complex of CBFβ factor (*yellow*) along with the runt domain (*orange*) and DNA (*green*)

have an effect on the other one. Here, mutation of Gly 192 to Arg induces a conformational rearrangement at loop 9 which in turn has direct hydrogen bonds with loop 5. The two loops move in a coordinated way. Although these loops are known to undergo conformational changes upon binding to CBFβ and DNA, we have superimposed the two most representative structures of the wild-type and mutant protein on the corresponding protein in complex with CBFβ and DNA (pdb id. 1h9d; Bravo et al 2001).

The side chain of Arg 192, in the mutant protein, still points toward DNA and steric hindrance with DNA could possibly be accommodated by the overall flexibility of this protein loop (Fig. 3b). However, the conformational changes needed at loop 9 in order to bind DNA are expected to have an impact on further CBFβ binding, through loop 5. This would explain why the mutant still retains some DNA binding capability but is not able to activate osteocalcin promoter.

Acknowledgements We thank Dr. G. Karsenty for providing plasmids pCMV-OSF2 and p6OSE2-luc. This work is funded by COFIN and Regione FVG grants to GD and GT and from Procter & Gamble to GT.

References

Bäckström S, Wolf-Watz M, Grundström C, Härd T, Grundström T, Sauer UH (2002) The RUNX1 runt domain at 1.25 Å resolution: a structural switch and specifically bound chloride ions modulate DNA binding. J Mol Biol 322:259–272

Bravo J, Li Z, Speck NA, Warren AJ (2001) The leukemia-associated AML1 (Runx1)–CBF beta complex functions as a DNA-induced molecular clamp. Nat Struct Biol 8:371–378

Damante G, Pellizzari L, Esposito R, Fogolari F, Viglino P, Fabbro D, Tell G, Formisano S, Di Lauro R (1996) A molecular code dictates sequence-specific DNA recognition by homeodomains. EMBO J 15:4992–5000

Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G (1997) Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89:747–754

Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M, Karsenty G (1999) A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Genes Dev 13:1025–1036

Fogolari F, Moroni E, Wojciechowski M, Baginski M, Ragona L, Molinari H (2005) MM/PBSA analysis of molecular dynamics simulations of bovine beta-lactoglobulin: free energy gradients in conformational transitions? Proteins 59:91–103

Gill G, Ptashne M (1988) Negative effect of the transcriptional activator GAL4. Nature 334:721–724

Lee B, Thirunavukkarasu K, Zhou L, Pastore L, Baldini A, Hecht J, Geoffroy V, Ducy P, Karsenty G (1997) Missense mutations abolishing DNA binding of the osteoblast-specific transcription factor OSF2/CBFA1 in cleidocranial dysplasia. Nat Genet 16:307–310

Mundlos S (1999) Cleidocranial dysplasia: clinical and molecular genetics. J Med Genet 36:177–182

Otto F, Kanegane H, Mundlos S (2002) Mutations in the RUNX2 gene in patients with cleidocranial dysplasia. Hum Mutat 19:209–216

Petrey D, Xiang Z, Tang CL, Xie L, Gimpelev M, Mitros T, Soto CS, Goldsmith-Fischman S, Kernytsky A, Schlessinger A, Koh IY, Alexov E, Honig B (2003) Using multiple structure alignments, fast model building, and energetic analysis in fold recognition and homology modeling. Proteins 53:430–435

Puppin C, D'Elia AV, Pellizzari L, Russo D, Arturi F, Presta I, Filetti S, Bogue CW, Denson LA, Damante G (2003) Thyroid-specific transcription factors control Hex promoter activity. Nucleic Acids Res 31:1845–1852

Quack I, Vonderstrass B, Stock M, Aylsworth AS, Becker A, Brueton L, Lee PJ, Majewski F, Mulliken JB, Suri M, Zenker M, Mundlos S, Otto F (1999) Mutation analysis of core binding factor A1 in patients with cleidocranial dysplasia. Am J Hum Genet 65:1268–1278

Tahirov TH, Inoue-Bungo T, Morii H, Fujikawa A, Sasaki M, Kimura K, Shiina M, Sato K, Kumasaka T, Yamamoto M, Ishii S, Ogata K (2001) Structural analyses of DNA recognition by the AML1/Runx-1 Runt domain and its allosteric control by CBFbeta. Cell 10:755–767

Tessa A, Salvi S, Casali C, Garavelli L, Digilio MC, Dotti MT, Di Giandomenico S, Valoppi M, Grieco GS, Comanducci G, Bianchini G, Fortini D, Federico A, Giannotti A, Santorelli FM (2003) Six novel mutations of the RUNX2 gene in Italian patients with cleidocranial dysplasia. Hum Mutat 22:104

Yoshida T, Kanegane H, Osato M, Yanagida M, Miyawaki T, Ito Y, Shigesada K (2003) Functional analysis of RUNX2 mutations in Japanese patients with cleidocranial dysplasia demonstrates novel genotype–phenotype correlations. Am J Hum Genet 71:724–738

Zhou G, Chen Y, Zhou L, Thirunavukkarasu K, Hecht J, Chitayat D, Gelb BD, Pirinen S, Berry SA, Greenberg CR, Karsenty G, Lee B (1999) CBFA1 mutation analysis and functional correlation with phenotypic variability in cleidocranial dysplasia. Hum Mol Genet 8:2311–2316