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Sp1/Sp3 and the myeloid zinc finger gene MZF1 regulate the human N-cadherin promoter in osteoblasts

S. Le Mée, O. Fromigué, P.J. Marie*

Laboratory of Osteoblast Biology and Pathology, INSERM U606, Lariboisière Hospital, 75475 Cedex 10 Paris, France

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Abstract

To determine the molecular mechanisms by which N-cadherin transcription is regulated, we cloned and sequenced a 3681-bp of the 5′-flanking region of the human N-cadherin gene. Deletion analysis of the proximal region identified a minimal 318-bp region with strong promoter activity in human osteoblasts. The cryptic promoter is characterized by high GC content and a GA-rich binding core that may bind zing finger transcription factors. Electrophoretic mobility shift assays (EMSA), competition and supershift EMSA revealed that an Sp1/Sp3 binding site acts as a basal regulatory element of the promoter in osteoblasts. Incubation of osteoblast nuclear extracts with −163/−131 wild-type probe containing the GA-rich binding core revealed another specific complex, which was not formed with a −163/−131 probe mutated in the GA repeat. EMSA identified the nuclear factor involved as myeloid zinc finger-1 (MZF1). Mutation analysis showed that Sp1/Sp3 and MZF1 binding sites contribute to basal promoter activity. Cotransfection analyses showed that Sp1 and MZF1 overexpression increases whereas Sp3 antagonizes Sp1-induced N-cadherin promoter activity in osteoblasts. RT-PCR analysis showed that human osteoblastic cells express MZF1 and that Sp1/MZF1 overexpression increased N-cadherin expression. These results indicate that Sp1/Sp3 and MZF1 are important transcription factors regulating N-cadherin promoter activity and expression in osteoblasts.
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Keywords: N-Cadherin; Sp1/Sp3; MZF1; Regulation; Promoter; Osteoblast; Differentiation

Introduction

Cadherins are single chain transmembrane glycoproteins that mediate calcium-dependent cell–cell adhesion [1,2]. Classic type I cadherins exhibit a conserved His-Ala-Val (HAV) motif in the first extracellular domain mediating homophilic interactions between cadherins, whereas type II cadherins lack the cell adhesion sequence motif HAV found in the N-terminal part of type I cadherins [2]. Cadherins are crucial for cell–cell interactions during early stages of morphogenesis and development [3,4] and thereby play essential roles in several physiological processes, including cell migration, proliferation, differentiation, and apoptosis [2,5]. Cadherins bind to intracellular proteins named catenins and the complex cadherins–catenins modulate a variety of processes including cell–cell adhesion as well as signal transduction and gene transcription [5]. Accordingly, abnormal cadherin expression was found to be associated with cancer cell migration, invasion, survival, and metastasis [6,7].

Cell–cell adhesion mediated by cadherins plays an important role in skeletogenesis [8,9]. During cartilage development, the early condensation events mediated by cell–cell adhesion involve N-cadherin and N-CAM expression [10,11]. In cells of the osteoblast lineage, cadherin-11 and N-cadherin are the more abundant cadherins expressed in vitro [12], and N-cadherin is strongly expressed in human osteoblasts in vivo [13]. Recent studies indicate that up-regulation of N-cadherin controls the expression of phenotypic genes in osteoblasts [13–15]. Consistently, suppression of N-cadherin expression in osteoprogenitor cells reduces cell–cell adhesion and expression of osteoblast genes [16,17], which suggests that basal N-cadherin expression may play a role in osteoblast differentiation and function. Although the mechanisms by which N-

* Corresponding author. Inserm U606, Lariboisière Hospital, 2 rue Ambroise Paré, 75475 Cedex 10 Paris, France. Fax: +33 1 49 95 84 52.
E-mail address: pierre.marie@larib.inserm.fr (P.J. Marie).

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cadherin expression regulates the differentiation of skeletal cells are still unclear, they may involve changes in cell–cell adhesion or alteration in β-catenin accumulation, resulting in the generation of intracellular signals and phenotypic gene expression [8,9,18].

A number of calciotropic factors, including glucocorticoids [19], tumor necrosis factor α, interleukin-1α [20], and transforming growth factor β [21] were found to regulate N-cadherin gene expression in skeletal cells. We recently reported the regulation of N-cadherin in human osteoblastic cells by bone morphogenetic-2 [22] and fibroblast growth factor-2 [23], two important regulators of bone formation. Additionally, recent data in chondrocytes suggest that N-cadherin is regulated by Wnt signaling, a pathway that plays a major role in the control of osteogenesis in humans [24]. Thus, several important regulatory pathways may converge to control the expression of the N-cadherin gene in skeletal cells. Understanding the transcriptional regulation of the human N-cadherin gene should therefore provide information on the molecular regulatory mechanisms involved in the differentiation of skeletal cells and other cells. However, little information is available on the regulation of the N-cadherin gene. The chicken N-cadherin promoter has been cloned and partially characterized [25]. Sequence analysis of this promoter region revealed no CAAT or TATA boxes, but showed a high overall GC content and several consensus Sp1 and AP-2 putative binding sequences. The molecular mechanisms involved in the functional regulation of the human N-cadherin gene are however not characterized.

Given the importance of N-cadherin in controlling skeletal cell differentiation, we examined the molecular mechanism by which basal N-cadherin transcription is regulated in human osteoblasts. In order to characterize the N-cadherin promoter and to identify regulatory elements, we isolated 3681 base pairs of 5′-flanking region of the N-cadherin gene from a human BAC library. Functional analysis established a minimal region involved in the regulation of the human N-cadherin promoter activity. Through EMSA, we identified transcription factors that regulate the basal activity of the human promoter. Mutational and gel shift analyses and transfection studies indicated that Sp1/Sp3 and myeloid zinc finger-1 (MZF1) regulate N-cadherin basal expression in human osteoblastic cells. Osteoblasts express MZF1 and overexpression of MZF1/Sp1 up-regulates N-cadherin expression in osteoblasts. The results show that multiple protein complexes including MZF1/Sp1 control N-cadherin promoter activity in osteoblasts.

Materials and methods

DNA subcloning and deletion mutants

Inserts were prepared using the restriction map of a BAC clone called 430E17 from a human BAC library RPCI-11 (Research Genetics, Groningen, The Netherlands), which covers the 5′-proximal region of human N-cadherin gene. The 3681-bp were subcloned into the promoterless pGL3 Basic vector (Promega, Charbonnières, France). Deletion fragments were generated by enzymatic digestion and gel-purified fragments (Qiaex II gel extraction kit, Qiagen, Courtaboeuf, France) were subcloned into pGL3 basic vector. For point mutation generation, a commercial kit (Quick Change mutagenesis kit, Stratagene, Amsterdam, The Netherlands) was used. Constructs and mutants were checked by sequencing.

Cell cultures

Immortalized human neonatal calvaria (IHNC) cells are human calvaria-derived osteoblastic cells showing phenotypic characteristics of osteoblasts [22,23]. Normal (Nl) cells are human immortalized osteoblastic cells [26]. A172 human glioma cells and SaOS2 human osteosarcoma cells were originally obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (292 mg/l), 10% heat-inactivated fetal calf serum (FCS), and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin).

Oligonucleotide probes

The oligonucleotides used for gel shift assays were synthesized (Sigma Genosys, Cambridgeshire, UK) and are indicated in Table 1. Equimolar amounts of single-stranded oligonucleotides were annealed, purified on 6% acrylamide gel, and radiolabeled using T4 polynucleotide kinase (Invitrogen, Cergy Pontoise, France) and [γ^{32}P]dATP. The radiolabeled probes were then separated from free nucleotides on MicroSpin G-50 Columns (Amersham Pharmacia Biotech Inc., Les Ulis, France).

Primer extension and RT-PCR analysis

Total RNA was extracted from IHNC cells by Extract-All reagent (Eurobio, Les Ulis, France). Primer extension reactions were performed using 50 μg of total RNA and the reverse primer 5′-GAGGCGGAGAGGGGCCGAGCGAA-GAGCCGG-3′. Primers were radiolabeled as described above, annealing and extension were performed as described in the Promega primer extension kit. After a phenol–chloroforme extraction, samples were ethanol precipitated and pellets were dissolved in 6 μl of loading buffer. The ϕX174 DNA/*Hin*fI dephosphorylated markers (Promega) were radiolabeled with [γ^{32}P]dATP. Manual sequencing of lambda gt11 control DNA was performed using the fmol DNA cycle sequencing system (Promega). The samples were denatured for 10 min at 90°C before loading on a 6% sequencing gel (Qbiogene, Illkirch, France). The gel was then vacuum dried and autoradiographed 1–2 days at −80°C.

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