Developmental association of the β-galactoside-binding protein galectin-1 with the nuclear matrix of rat calvarial osteoblasts

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SUMMARY

The protein composition of the nuclear matrix changes significantly as the osteoblast matures from a proliferating pre-osteoblast to an osteocyte embedded in a mineralized matrix. These matrix proteins are the result of developmental stage-specific gene expression during osteoblast differentiation. To isolate nuclear matrix proteins unique to the bone phenotype we analyzed nuclear matrix preparations from cultures of rat calvarial osteoblasts by high resolution two-dimensional gel electrophoresis at two different stages: proliferation (day 3) and differentiation (day 18, mineralized). We characterized one protein (14 kDa; pI 5.0), that was detectable only in the nuclear matrix of differentiated osteoblasts. By mass spectrometry and microsequencing, this protein was identified as the β-galactoside-binding protein galectin-1. Both immunofluorescence staining of nuclear matrix preparations with the galectin-1 antibody and western blot analysis of subcellular fractions confirmed that galectin-1 is only associated with the nuclear matrix in differentiated osteoblasts as the result of differential retention. Galectin-1 protein and mRNA are present throughout osteoblast differentiation. Galectin-1 is present in the cytoplasmic and nuclear fractions in both proliferating and differentiated osteoblasts. However, its only stable binding is to the nuclear matrix of the differentiated osteoblast; but, in proliferating osteoblasts, galectin-1 is not retained in the nuclear matrix. Taken together, our results suggest that developmental association of galectin-1 with the nuclear matrix reflects differential subnuclear binding of galectin-1 during osteoblast differentiation.

Key words: Galectin-1, Osteoblast, Nuclear matrix, Differentiation

INTRODUCTION

The machinery of gene regulation is associated with nuclear architecture during cellular growth and differentiation (Jackson, 1997). The nuclear matrix (Berezney and Coffey, 1974) is the underlying structure that remains after removal of chromatin. It consists of the nuclear pore-lamina complex and the internal non-chromatin nuclear structures. The nuclear matrix-intermediate filament (NM-IF) scaffold maintains the shape and intracellular position of the nucleus (Penman et al., 1997). The NM-IF supports important nuclear functions such as DNA replication (Cook, 1991), transcription (Stein et al., 1997), RNA processing and nuclear transport (Blencowe et al., 1994). However, only a few specific nuclear matrix proteins have been identified (Kaufmann et al., 1986; Guo et al., 1995; Merriman et al., 1995; Xing et al., 1995; Mattern et al., 1997).

The nuclear matrix is composed of at least two classes of proteins, those that are completely or relatively ubiquitous and a population of cell-type specific or developmentally associated proteins (Fey and Penman, 1988; Getzenberg, 1994; Bidwell et al., 1993; Lindenmuth et al., 1997). For example, B23 and hnRNP proteins are common, ubiquitous nuclear matrix proteins (Stuurman et al., 1990; Mattern et al., 1997). In contrast, the CBF/AML family of transcription factors are examples of cell type-specific nuclear matrix proteins (Merriman et al., 1995; Zeng et al., 1997). Modifications in protein composition of the nuclear matrix have been observed during differentiation (Stuurman et al., 1989; Dworetzky et al., 1990) and transformation (Getzenberg et al., 1991; Bidwell et al., 1994a). In addition, the composition of the nuclear matrix is altered in response to physiological mediators of bone-tissue specific gene expression (Bidwell et al., 1994b, 1996). Thus, the composition of the nuclear matrix responds dynamically to the intra- and extracellular environment. These modifications of nuclear matrix protein composition may contribute to the process of differentiation.

In previous studies, we have shown that osteoblasts express bone-related proteins as well as phenotype-specific nuclear matrix proteins during osteoblast differentiation (Owen et al., 1990; Dworetzky et al., 1990; Bidwell et al., 1993; Merriman et al., 1995; Lindenmuth et al., 1997; Banerjee et al., 1997). In this study, we have further analyzed proteins that are developmentally associated with the nuclear matrix in primary cultures of rat calvarial osteoblasts. We used preparative high
resolution two-dimensional gel electrophoresis to isolate proteins that differ markedly in their amounts at two stages of the osteoblast developmental sequence. We identified galectin-1 as a stage-specific nuclear matrix protein in differentiated osteoblasts. By western blot and immunofluorescence analyses we show that galectin-1 associates with the nuclear matrix during development of the osteoblast phenotype.

MATERIALS AND METHODS

Cell culture

Calvaria from fetal rats of 21 days gestation were isolated and subjected to sequential digestion for 20, 40, and 90 minutes at 37°C in 2 mg/ml collagenase (Boehringer Mannheim, Indianapolis, IN, USA) with 0.25% trypsin (Gibco BRL, Grand Island, NY, USA) (Owen et al., 1990). Primary osteoblasts from the third digestion were plated in minimal essential medium (MEM; Gibco BRL) supplemented with 10% fetal calf serum (FCS) and dialyzed (2,000 molecular mass cutoff) at 4°C against

Two-dimensional gel electrophoresis

High-resolution two-dimensional (2-D) gel electrophoresis was performed according to the method of O’Farrell (1975) using the Millipore InvestigatorTM 2-D electrophoresis system (Millipore, Bedford, MA, USA). The amount of protein was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Electroblotting, mass spectrophotometry and microsequencing

A 17 cm × 19 cm section of the gel was transferred onto an Immobilon PSQ membrane (Millipore) by semidyry electroblotting (OWL Scientific Plastics, Cambridge, MA, USA). The transfer assembly stack matrix was constructed with a Whatman 3M filter equilibrated with a buffer containing 0.375 M Tris, pH 9.4, 3% SDS, and 0.01% Bromphenol Blue. The gel was layered on top of SDS-12% polyacrylamide slab gels. The second dimension electrophoretic separation was performed in 10% T, 2.6% C polyacrylamide gels. Proteins were separated in the first dimension by isoelectric focusing (IEF) in polyacrylamide gels containing 4% T, 2.6% C (18 cm × 3 mm i.d.). The IEF gel also contained 8 M urea, 2% octyl-D-glucopyranoside and 5.5% carrier 3-10/2D ampholytes. Gels were polymerized for 1 hour by addition of 78 µl 10% ammonium persulfate (w/v) and 25 µl of TEMED (v/v) to 25 ml of gel solution. Samples containing 1 mg of proteins (100-200 µl) were applied directly to the gel after 30 µl of overlay buffer. IEF gels were run for a total of 17,500 volt-hours; anode and cathode solutions were 0.08 M phosphoric acid and 0.1 M NaOH, respectively. After focusing, the gel was extruded with a syringe as recommended by the manufacturer and equilibrated for 20 minutes in a buffer containing 0.375 M Tris, pH 9.2, 3% SDS, and 0.01% Bromphenol Blue. The tube gels were layered on top of SDS-12% polyacrylamide slab gels. The second dimension electrophoretic separation was performed in 10% T, 2.6% C polyacrylamide gels. The second dimension electrophoretic separation was performed in 10% T, 2.6% C polyacrylamide gels.

Electroblotting was performed at 250 mA for 90 minutes at 20°C using a fixed-angle centrifuge at 150,000 g for 95 minutes at 20°C using a fixed-angle rotor (Beckman) to pellet the reassembled intermediate filaments. The supernatant containing the nuclear matrix proteins was concentrated using Centricon-10 tubes (Amicon, Beverly, MA, USA). The concentrate was mixed with sample buffer according to the protocol of the Millipore Investigator™ 2-D electrophoresis system (Millipore, Bedford, MA, USA). The amount of protein was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard.

Electroblotting, mass spectrophotometry and microsequencing

A 17 cm × 19 cm section of the gel was transferred onto an Immobilon PSQ membrane (Millipore) by semidyry electroblotting (OWL Scientific Plastics, Cambridge, MA, USA). The transfer assembly stack matrix was constructed with a Whatman 3M filter equilibrated in anode buffer 1 (250 mM Tris, pH 10.4, 10% v/v methanol), two sheets equilibrated in anode buffer 2 (25 mM Tris, pH 10.4, 10% v/v methanol). Immobilon PSQ membrane equilibrated in anode buffer 1, the gel equilibrated in cathode buffer (25 mM Tris, pH 9.4, 20% v/v methanol, 40 mM glycine, 0.05% w/v SDS), and three sheets of filter paper equilibrated in cathode buffer. Electroblotting was performed at 2 mAcm² for 60 minutes. The membrane was stained with Amido Black according to the method of Sánchez et al. (1992) and then briefly rinsed in water until background staining did not interfere with pattern recognition. At 2°C. Protein excised from 2-3 Immobilon PSQ membranes was digested in situ with trypsin in a digest buffer containing 100 mM ammonium bicarbonate, 10% acetoniitrile, and 1% octylglucoside (Gharahdaghi et al., 1996). The digest mixture was separated on a 0.5 mm × 150 mm capillary C18 column (Applied Biosystems, Foster City, CA, USA) which was coupled to an LC Packings (San Francisco, CA, USA) flow splitter on a modified Hewlett Packard 1090 M HPLC system. Peptides were eluted using a linear gradient from 100% solvent A (0.1% trifluoroacetic acid in water) to 46% solvent B (0.08% trifluoroacetic acid in acetoniitrile/water: 70/30) at a flow rate of 20 µl/minute. The eluent was monitored at 210 nm and fractions collected manually. An aliquot (0.5 µl) of each peptide was subjected to Matrix-Assisted-Laser Desorption Time-of-Flight mass spectrometry (MALD-TOF) using a Perseptive Biosystems.
Cloning of rat galectin-1

On the basis of the reported cDNA sequence of galectin-1 (Clerch et al., 1988) two primers each with either a KpmI or EcoRV site (upstream primer; 5'-GGCGGTACCAATGGCCTGTGGTCTGGTCG-3' and downstream primer; 5'-GCGATTCAAAAAAGCCCAACACTTAT-3'), respectively, were prepared using a Beckman Oligo 1000M DNA synthesizer (Beckman, Fullerton, CA, USA). The polymerase chain reaction with reverse transcription (RT-PCR) was carried out using these primers with rat osteoblast RNA (day 18). The correct single PCR product (421 bp) was purified by Qiagen Gel extraction kit (Qiagen, Santa Clara, CA, USA).

Northern blot analysis

Total RNA was isolated from rat calvarial osteoblasts using TrizolR (Gibco-BRL) according to the manufacturer’s specifications. RNA (10 μg per lane) was separated in a 1% agarose-formaldehyde gel, transferred onto Zetaprobe membrane (Bio-Rad Labs, Hercules, CA, USA) and hybridized to probes for rat alkaline phosphatase (Noda and Rodan, 1987), rat osteocalcin (Lian et al., 1989) and rat galectin-1 (cloned in this study). Hybridization was performed at 68°C and the blot washed extensively in buffer containing 0.1% SSC and 0.1% SDS at 55°C. Messenger RNA abundance was quantitated by using a Storm 840 Phosphoimager (Molecular Dynamics, Sunnyvale, CA, USA).

Western blot analysis

For whole cell extract, cell layers were washed with ice-cold phosphate-buffered saline, scraped, centrifuged and lysed in a 50 mM Tris-HCl buffer, pH 7.4, containing 0.25 M NaCl, 0.5% (v/v) Nonidet P40 (NP40), 20 μg/ml TPCK, 20 μg/ml trypsin inhibitor, 10 μg/ml leupeptin and 1 mM PMSF. The cells were then homogenized on ice in a tight-fitting dounce homogenizer (35 strokes), centrifuged at 13,000 g for 10 minutes at 4°C and the supernatant was stored at −70°C. For analysis of specific subcellular fractions during nuclear matrix protein preparations, cells were sequentially extracted with the same buffers as described for nuclear matrix preparations, except for the digitonin procedure. For the digitonin procedure, cells were treated with digitonin as described by Moore and Blobel (1992). The supernatant and pellets were stored until use and pellets were extracted further to prepare nuclear matrix protein. Equal amounts of protein (30 μg) from each fraction were separated by SDS-PAGE and then transferred to Immunobilon PVDF membrane (Millipore). The membranes were blocked with 0.5% nonfat milk for 1 hour, incubated with antibodies for 1 hour, and reactive bands were visualized using the ECL detection system (Amersham). Galectin-1 antibody was a kind gift from Dr Richard D. Cummings, University of Oklahoma, USA (Cho and Cummings, 1995). The dynin antibody was a generous gift from Dr Richard V aughan (University of Massachusetts Medical School) (V aughan and V allee, 1995). Polyclonal lamin B antibody was purchased from Santa Cruz, CA, USA. Protein abundance was quantitated by analysis of western blots using densitometry (Molecular Dynamics). Total protein concentration in the cell lysate was determined using the Micro BCA protein assay kit (Pierce).

Immunofluorescence staining and image analysis

Cells were prepared for immunofluorescence analysis by using the stabilized nuclear matrix protocol (Nickerson et al., 1997). Briefly, cells were washed in cold PBS. Soluble proteins were removed by extraction in CSK-2 buffer (CSK buffer containing 20 mM vanadyl riboside complex and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) for 5 minutes on ice. For the CSK preparation, the structure remaining was extensively cross-linked by treatment with 3.7% formaldehyde in CSK-2 for 10 minutes. For NM-IF analysis, cells were washed further in CSK-2, and then digested with DNA 400 units/ml DNase I (Boehringer Mannheim) for 1 hour at room temperature in digestion buffer (DB: DIG buffer containing 20 mM vanadyl riboside complex and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) to remove DNA and associated proteins. Digestion was stopped by addition of ammonium sulfate to final 0.25 M in DB solution and washed twice with DB solution for 5 minutes each. The NM-IF was fixed with 3.7% formaldehyde in PBS. The same polyclonal antibody against hamster galectin-1 described for western blot analysis was used. Optimal concentration was determined through serial dilution of primary antibody (data not shown). Omission of the primary antibodies resulted in complete loss of the corresponding cytoplasmic and nuclear signals. DNA content of whole cells and nuclear matrix preparations was assessed by staining with 4,6-diamidino-2-phenyl indole (DAPI) (Coleman et al., 1981).

RESULTS

Identification of stage-specific nuclear matrix proteins during osteoblast differentiation

Rat primary calvarial osteoblasts are well characterized with regard to the temporal and spatial expression of genes encoding bone-related proteins during osteoblast differentiation (Owen et al., 1990). To identify nuclear matrix proteins that associate with the developmental expression of genes in calvarial osteoblasts and to allow for possible variations in the osteoblast developmental sequence, we isolated three independent nuclear matrix protein extracts from two developmental stages: the proliferating (day 3) and differentiated stage (day 18). We confirmed that the differentiated osteoblasts analyzed in this study were characterized by an abundance of alkaline phosphatase positive cells, increased osteocalcin biosynthesis and mineralizing nodules (Stein et al., 1990). NM-IF preparations were performed by sequential detergent extraction and nuclease digestion. Protein fractions were enriched for (non-structural) nuclear matrix proteins by solubilizing the NM-IF with urea and removal of intermediate filaments by ultracentrifugation. To evaluate reproducibility of independent experiments, multiple nuclear matrix protein preparations were electrophoresed simultaneously and no differences between preparations were observed in one dimensional protein profiles. However, electrophoretic patterns of nuclear matrix proteins from proliferating and differentiated stages were distinct. These observed modifications in nuclear matrix protein profiles extend the original finding by Dworetzky et al. (1990).

High resolution 2-D gel electrophoresis consistently demonstrated differences in nuclear matrix protein profiles (Fig. 1) between the proliferation and differentiation stages. One group of nuclear matrix proteins is detectable at both stages, although the relative representation may differ between proliferation and differentiation stages. Other classes of nuclear matrix proteins are detectable in either proliferating or differentiated osteoblasts. Inspection of multiple 2-D gels containing nuclear matrix proteins isolated at the two developmental stages led to the identification of a protein

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present in the day 18 nuclear matrix protein preparations with a molecular mass of 14 kDa and pI 5.0 (Fig. 1B, arrow). This 14 kDa protein, abundant in the nuclear matrix at the differentiation stage, appears to be a bone-related nuclear matrix protein. Therefore we isolated and microsequenced this protein, and determined its identity.

**Galectin-1 is a nuclear matrix protein in differentiated osteoblasts**

To identify the 14 kDa protein, the electroblotted spot was excised from the Immobilon PSQ membrane and digested in situ with trypsin (see Materials and Methods). The resultant peptides were resolved by HPLC and selected fractions analyzed by MALD-TOF mass spectrometry. A single fraction was then microsequenced. When analyzing the mass spectral data, the search was restricted to tryptic digests of rat proteins in the 1,000 to 30,000 Da range using a 3 Da tolerance. Analysis of peptide molecular mass profiles revealed the greatest similarity with the β-galactoside-binding protein, galectin-1 (Table 1). Amino acid sequence analysis of the 1,805 Da peptide fragment provided 16 residues of sequence (LNMEAINYMAADGDFK) consistent with the mass spectrometry data. A computer search of the NCBI nonredundant data base using the BLAST program showed a

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**Table 1. Identification of a 14 kDa protein by microsequencing**

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<td>1501.6699</td>
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(A) Six tryptic fragments were analyzed by mass spectrometry. An MS-Fit search using these six peptide masses indicated a perfect match with rat galectin-1. Met-ox and Acet N are oxidation of methionine and N terminus acetylated, respectively. (B) Protein sequence of galectin-1 as retrieved from GenBank (accession no. M19036). One peptide fragment (1,805 Da) which was microsequenced is underlined.
100% match with galectin-1. The electrophoretic properties of galectin-1 (molecular mass 14 kDa and pI 5.0) matched the result of our 2-D gel electrophoretic analysis. Hence, the mass spectrometry fingerprint and sequence analysis provide consistent outcomes and demonstrate that the 14 kDa protein isolated from the nuclear matrix of differentiated osteoblasts is galectin-1.

### Expression of galectin-1 mRNA during osteoblast differentiation

The 2-D gel electrophoresis results show that galectin-1 is present only in the nuclear matrix of the differentiated and not the proliferating osteoblasts. To address whether galectin-1 expression is developmentally regulated during osteoblast differentiation, we performed northern blot analysis with total RNA prepared at several time points from growth to differentiation stages of cultured rat calvarial derived osteoblasts (Fig. 2). For these studies, we cloned the full length galectin-1 cDNA by RT-PCR. The sequence of this clone is identical to the rat lung and uterus galectin-1 (Clerch et al., 1988) and different from the activated macrophage type galectin-1 like protein (Rabinovich et al., 1998). Using galectin-1 cDNA as a probe, a 0.65 kb transcript was detected by northern blot analysis. We found that galectin-1 mRNA was expressed in proliferating osteoblasts (day 5), and that the mRNA levels increased only 2-fold by day 22 during mineralization. Subsequently galectin-1 mRNA levels declined between day 25 and day 29 (Fig. 2) with the onset of apoptosis (Lynch et al., 1997) during the later stage of the osteoblast culture period. The expression of other bone-related mRNAs, such as alkaline phosphatase and osteocalcin, indicating mature osteoblasts, appeared as in previous studies (Owen et al., 1990). These results suggest that galectin-1 mRNA levels are minimally regulated during osteoblast differentiation and that other mechanisms must account for the observed differentiation-specific presence of galectin-1 in the nuclear matrix.

### Differential partitioning of galectin-1 between the nuclear matrix and non-matrix compartments during bone cell phenotype development

We directly addressed whether the galectin-1 protein level or the subcellular distribution of galectin-1 is modified during osteoblast differentiation. We performed western blot analysis with whole cell and nuclear matrix proteins which were extracted at days 3 (proliferating), 11 or 14 (matrix maturation), and 21 (mineralization) (Fig. 3). When proteins from whole cell lysates (WC) were assayed, galectin-1 was detected on day 3 and increased approximately 2-fold by day 21 (Fig. 3). This result is consistent with our northern blot analysis showing that galectin-1 mRNA is expressed at similar levels in both proliferating and differentiated osteoblasts. However, galectin-1 was detected only in the nuclear matrix protein fraction (NM) of differentiated osteoblasts, a result similar to the 2-D gel electrophoresis results presented in Fig. 1. As an internal control, we assessed the presence of dynein and lamin B. As expected, dynein, which is present predominantly in the cytoplasm, is detectable in the whole cell extract but undetectable in the nuclear matrix fractions. In contrast, lamin B, which is a nuclear matrix component, is enriched in the nuclear matrix fraction and barely detectable in whole cell fractions. These results show that the nuclear matrix proteins obtained by biochemical fractionation are not contaminated with cytoplasmic proteins. Thus, our findings strongly suggest that subnuclear distribution of galectin-1 is developmentally regulated during osteoblast differentiation.

**Fig. 2.** Developmental expression of galectin-1 during osteoblast differentiation. Total RNAs were prepared from second passage rat calvarial osteoblast cultures at the indicated days. Representation of mRNAs was analyzed by northern blot. RNA (10 µg) was hybridized with galectin-1, alkaline phosphatase (ALP) and osteocalcin cDNAs as probes. The autoradiogram was developed as described in the Materials and Methods. Each probe was used sequentially for hybridization with the same nylon membrane after removal of the previous signal.

**Fig. 3.** Developmental association of galectin-1 with the nuclear matrix during osteoblast differentiation. Western blot analyses were performed on protein preparations obtained from rat osteoblasts at different stages of growth (day 3) and differentiation (days 11, 14, 21). Samples (30 µg per lane) were resolved in 12% polyacrylamide gels and blotted onto PVDF membrane. Blots were incubated with the antibody for galectin-1 and the signal was detected by chemiluminescence. Subsequently, the same membranes were stripped and reprobed with antibodies for lamin B (67 kDa) or dynein (75 kDa). Whole cell (WC) and nuclear matrix (NM) fractions were prepared as described in Materials and Methods.
cells was very similar (data not shown). We then examined remaining pellet from the Triton X-100 or digitonin-treated galectin-1 immunoreactivity in the soluble fraction and with either digitonin or Triton X-100. The distribution of lamin B staining is observed in cells treated with Triton X-100 immunofluorescence staining (Fig. 4). Fig. 4A shows that validated that the digitonin procedure does not permeabilize localization in the same experiment (Fig. 4). Initially, we of digitonin and Triton X-100 treatment on galectin-1 described (Moore and Blobel, 1992). We compared the effects plasma membrane but not the nuclear membrane as previously matrix (Cho and Cummings, 1995; Cuperlovic et al., 1995; Akimoto et al., 1995). In addition, as galectin-1 is a small protein (14 kDa), the possibility arises that biochemical fractionation which involves an extraction with a Triton X-100 containing buffer may result in relocalization of galectin into the nucleus due to disruption of the nuclear membrane. Therefore, we performed a different extraction procedure, using the detergent digitonin, which will only permeabilize the plasma membrane but not the nuclear membrane as previously described (Moore and Blobel, 1992). We compared the effects of digitonin and Triton X-100 treatment on galectin-1 localization in the same experiment (Fig. 4). Initially, we validated that the digitonin procedure does not permeabilize the nuclear membrane of osteoblasts, by examining lamin B immunofluorescence staining (Fig. 4). Fig. 4A shows that lamin B staining is observed in cells treated with Triton X-100 buffer, but not in digitonin treated cells.

We then carried out western blot analysis of cells treated with either digitonin or Triton X-100. The distribution of galectin-1 immunoreactivity in the soluble fraction and remaining pellet from the Triton X-100 or digitonin-treated cells was very similar (data not shown). We then examined nuclear matrix proteins prepared from digitonin or Triton X-100 treated proliferating and differentiated osteoblasts (Fig. 4B). Although soluble cytoplasmic galectin-1 was extracted by digitonin treatment before permeabilization of the nucleus with Triton X-100 (NM-D), we still detected galectin-1 in the nuclear matrix preparation of differentiated osteoblasts (but not proliferating osteoblasts). Whole cells (WC) and the nuclear matrix intermediate filament (NM-IF) preparation which corresponds to the procedure used in immunofluorescence studies (see below) are shown for comparison. The representation of galectin-1 is similar to that observed in standard NM (NM-T) and NM-IF preparations (which were not pretreated with digitonin). These results indicate that Triton X-100 does not cause the relocalization of galectin-1 in rat osteoblasts. Taken together, these findings demonstrate galectin-1 is associated with the nuclear matrix in differentiated osteoblasts.

**Immunofluorescence analysis of the developmental association of galectin-1 with the nuclear matrix during osteoblast differentiation**

We also examined the subcellular distribution of galectin-1 by immunofluorescence to verify that association of galectin-1 with the nuclear matrix is developmentally regulated in osteoblasts. Fixed whole cells and cells extracted to obtain the NM-IF were compared (Fig. 5). In whole cells, galectin-1 staining was detected in the cytoplasm and abundantly in the nucleus at both proliferation and differentiation stages. The higher background staining in the differentiation stage reflects a cumulative signal from the multiple layers of osteoblasts in the cultures. In the CSK preparations (Triton X-100 extraction), the nuclei of differentiated osteoblasts stain intensely compared to proliferating cells. Thus, a much greater percentage of galectin has been extracted from the nuclei of proliferating osteoblasts. The NM-IF preparation indeed shows that galectin-1 is retained in the nucleus of differentiated osteoblasts. Although it is difficult to quantify the amount of protein by immunofluorescence in situ, the intensity of the signal in both CSK and NM-IF preparations from differentiated osteoblasts (day 14) is stronger than from proliferating osteoblasts (day 3). These results provide direct evidence that galectin-1 is a nuclear matrix protein and that the association of galectin-1 with the nuclear matrix is developmentally regulated during osteoblast differentiation. Taken together, these observations by immunofluorescence are consistent with the biochemical identification of galectin-1 as a nuclear matrix protein in differentiated osteoblasts.

**DISCUSSION**

In this study, we characterized a protein that is developmentally associated with the nuclear matrix during osteoblast differentiation. Using mass spectrometry and peptide sequencing, this nuclear matrix protein was identified as the β-galactoside-binding protein, galectin-1. The combined results from northern blot analysis, subcellular fractionation, and immunofluorescence analysis of the nuclear matrix in situ reveal a temporal association of galectin-1 with the nuclear matrix in mature osteoblasts.

The subcellular distribution of galectins is diverse. Galectin-
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1 has been observed in the nucleus, cytoplasm, cell surface, and the extracellular matrix (Akimoto et al., 1995; Cuperlovic et al., 1995; Cho and Cummings, 1995). In this study, we have shown that in proliferating osteoblasts which do not express bone-specific phenotypic markers (Stein and Lian, 1993), galectin-1 is present in both the cytoplasm and the nucleus, but is not tightly bound to the nuclear matrix. In differentiated osteoblasts, some galectin-1 is detected in the cytoplasm, but its primary location is throughout the nucleus and most is tightly associated with the nuclear matrix. Taken together, the change in binding to the nuclear matrix in concert with cellular differentiation suggests that the subcellular localization of galectin-1 is regulated in a developmental stage-specific manner and may support a specialized function of differentiated osteoblasts.

Galectins and related lectins may support interaction of a broad spectrum of glycoproteins with the nuclear matrix. In the galectin family (Barondes et al., 1994), galectin-3 has also been shown to be associated with the nuclear matrix (Wang et al., 1995). Furthermore, galectin-3 is expressed in hypertrophic chondrocytes (Fowlis et al., 1995) and osteoblasts (Aubin et al., 1996). Galectins may be functionally redundant as is suggested by the observation that galectin-1 and galectin-3 knockout mice show no special phenotypic changes (Poirier and Robertson, 1993; Colnot et al., 1998). The multifunctionality of galectins is reflected by their involvement in transformation (Yamaoka et al., 1991), cell growth (Wells and Mallucci, 1991), apoptosis (Perillo et al., 1995) and cell-cell and cell-matrix interactions in many tissues (Cooper et al., 1991; Baum et al., 1995). Galectins, as well as other nuclear lectins (Felin et al., 1997), may represent acceptor proteins for compartment specific glycoproteins.

A long standing observation is that glycoproteins and glycosaminoglycans are integral components of the nucleus (Stein et al., 1975; Hart et al., 1989; Hubert et al., 1989). Interestingly, many transcription factors including SpI (Jackson and Tjian, 1988), serum response transcription factor (Reason et al., 1992), and RNA polymerase II (Kelly et al., 1993) are glycoproteins. Furthermore, we and others have shown that transcription factors can partition differentially between the nuclear matrix and non-matrix compartments in a cell type, cell growth, and/or developmental stage-specific manner (van Wijnen et al., 1993; Lindenmuth et al., 1997). The possibility arises that galectins and other glycoprotein binding factors may function to tether gene regulatory factors to the nuclear matrix.

It is well established that the nuclear matrix supports transcriptional and posttranscriptional regulatory events, including RNA splicing (Blencowe et al., 1998). Interestingly, galectin-1 and galectin-3 have each been shown to be involved in pre-mRNA splicing (Vyakarnam et al., 1997; Dagher et al., 1995; Wang et al., 1995). The redistribution of galectin-1 to the nuclear matrix in differentiated osteoblasts suggests that the cellular role of galectin-1 is altered during osteoblast differentiation. The selective association of galectin-1 with the nuclear matrix in differentiated osteoblasts is consistent with its potential involvement in the transcriptional and posttranscriptional regulation of tissue-specific genes. The selectivity for these functions may be mediated in part by the specific subcellular location of galectin-1 and its cognate glycoprotein(s) during osteoblast differentiation.

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![Fig. 5. Immunofluorescence in situ analysis of galectin-1 in both proliferation and differentiation stage osteoblasts. Immunofluorescence analysis of rat calvarial osteoblasts was performed with a galectin-1 primary antibody and a Texas Red-conjugated secondary antibody. Whole cells (WC) (top panels) were sequentially extracted in situ with CSK buffer (middle panels), and digestion buffer to obtain the nuclear matrix intermediate filament (NM-IF) (bottom panels). Proliferating (left panels; day 3) and differentiated (right panels; day 14) cells were compared. DAPI staining identifies the nucleus for each antigalectin-1 stained panel. The micrographic exposures and magnifications (×1,000) are identical for all panels.](image-url)
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