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Proline-Rich Transcript of the Brain (*prtb*) Is a Serum-Responsive Gene in Osteoblasts and Upregulated During Adhesion

Dirk W. Sommerfeldt,[1] Jizu Zhi,[2] Clinton T. Rubin,[2,3] and Michael Hadjiargyrou[1,2]*

[1]Department of Orthopaedics, State University of New York at Stony Brook, Stony Brook, New York 11794-2580
[2]Department of Biomedical Engineering, State University of New York at Stony Brook, Stony Brook, New York 11794-2580
[3]Center for Biotechnology, State University of New York at Stony Brook, Stony Brook, New York 11794-2580

Abstract To characterize the temporal expression of genes that play a functional role during the process of osteoblast adhesion, we used differential display (DD-PCR) on mRNA isolated from attached vs. suspended osteoblasts. A 200-bp fragment displaying upregulated expression after 30 and 60 min adhesion was isolated, sequenced, and showed 97% homology to *prtb*, previously showed to be expressed in mouse brain. Northern analysis confirmed a two-fold increase in *prtb* message during adhesion to tissue culture polystyrene, both in the presence or absence of surface-adsorbed serum proteins. Serum stimulation alone was also able to induce *prtb* expression, although to a lesser extent, in suspension cells. Strong *prtb* expression was also detected in both brain and bone of adult rats. Furthermore, *prtb* expression analysis during MC3T3-E1 cell differentiation revealed high expression levels independent of proliferation (day 0–7), matrix maturation (day 7–14), and mineralization (day 14–31). Time course analysis of *prtb* expression during adhesion of sensitized osteoblasts to serum-protein coated surfaces showed robust mRNA expression at 5 min post-plating and a peak at 10 min. The two known serum-inducible immediate early genes *c-fos* and *c-jun* showed similar expression kinetics, with *c-jun* mRNA levels peaking at 15 min and *c-fos* at 20 min. Based on these data, we hypothesize that *prtb* may function as an immediate early, serum-responsive, and adhesion-inducible gene with possible involvement in processes such as cell cycle control, adhesion, and proliferation. J. Cell. Biochem. 84: 301–308, 2002.

Key words: *prtb*; osteoblast; adhesion; serum; mRNA; expression

Cellular attachment to extracellular matrix (ECM) proteins is an essential mechanism in cell migration, condensation, differentiation, and the prevention of apoptosis in anchorage-dependent cell types such as osteoblasts, fibroblasts, and cells from many other differentiated tissues [Hall and Miyake, 1995]. The osteoblast as an anchorage-dependent cell is extremely sensitive to signals from its microenvironment. For example, condensation of osteoprogenitor cells during embryonic development is controlled by feedback signal transduction between the bone-forming cells and the ECM [Franceschi, 1999]. Cell-matrix interactions also play a critical role during fracture healing and bone remodeling [Bostrom et al., 1995; Stenbeck and Horton, 2000]. These findings support the hypothesis that it might be possible to exert control over basic cellular functions such as proliferation and differentiation via the extracellular matrix [Bruder and Fox, 1999; Garcia et al., 1999].

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Dirk W. Sommerfeldt's present address is University Hospital Hamburg, Department of Trauma and Reconstructive Surgery, Martinistrasse 52, 20246 Hamburg, Germany.

*Correspondence to: Michael Hadjiargyrou, PhD, Department of Biomedical Engineering, Psychology A, 3rd Floor, State University of New York at Stony Brook, Stony Brook, NY 11794-2580. E-mail: michael.hadjiargyrou@sunysb.edu

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In the context of biomaterials research, osteoblast adhesion has been thoroughly studied on a morphological level, but very few studies have analyzed the underlying mechanisms on a molecular level [Kato et al., 1998, 2000; Zreiqat et al., 1999]. Adhesion itself seems to be a trigger for many basic cellular functions and non-adherent osteoblasts undergo apoptosis within 48 h [Damsky, 1999]. A better understanding of these regulatory mechanisms during osteoblast adhesion could lead to desirable control over basic cellular functions such as proliferation and/or differentiation, e.g., in the context of tissue formation in vitro.

In order to identify genes regulated via osteoblast adhesion to a substrate, we hypothesized that these genes should be differentially expressed between adherent and suspended cells at various timepoints. Consequently, DD-PCR [Liang and Pardee, 1992] was utilized as a means to identify such genes. The identification and isolation of *prtb* [Yang and Mansour, 1999], a highly conserved known gene coding for a proline-rich cytosolic protein is described here, as well as its expression during differentiation of MC3T3-E1 mouse osteoblastic cells and in intact normal bone tissue. We further show that *prtb* expression during osteoblast adhesion to adsorbed serum proteins exhibits similar temporal kinetics as the immediate-early serum-responsive genes *c-fos* and *c-jun*.

MATERIALS AND METHODS

Cell Culture, Differentiation, and Adhesion Experiments

MC3T3-E1 [Sudo et al., 1983] cells were maintained in log growth phase using α-MEM supplemented with 10% FBS (Life Technologies, Grand Island, NY). For differentiation studies, 50 μg/ml of ascorbic acid and 10 mM β-glycerol phosphate were added. Medium was changed every 3 days and cells were harvested on day 2, 4, 7, 10, 13, 16, 19, 22, 25, 28, and 31. For adhesion studies, tissue culture plates (TCP) were pretreated with 10% serum-containing media (TCP_{pre}) or serum-free media for 1 h at 37°C. Sensitized quiescent cells (serum-starvation for 18 h) were trypsinized using 0.01% trypsin and 5 mM EDTA for 2 min at 37°C. Trypsinization was terminated using soybean trypsin inhibitor (Sigma Chemicals, St.Louis, MO), and cells were maintained in a serum-free recovery media containing 0.1% BSA for 1 h prior to plating. Cells were then counted and plated out on 35-mm petri dishes at a density of $10^5/cm^2$ in serum-free media. Similarly, cells maintained in suspension were either induced with 10% FBS containing media for 30 min or kept in suspension without serum. After 30 min of attachment, floating cells were washed off with PBS, photomicrographs taken at 20 × magnification, and adherent cells were lysed in lysis buffer containing 1% β-mercaptoethanol on the plate. Cells in suspension were lysed accordingly.

RNA Isolation

Total RNA was isolated from cells using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Briefly, adherent cells were gently washed three times with warm PBS, lysed in the dish using lysis buffer containing 1% β-mercaptoethanol. The lysate was homogenized through a 21G needle and transferred to an RNeasy mini spin column. It was then passed through the column, and captured total RNA was washed three times using different washing buffers according to the protocol. After drying the RNeasy membrane, total RNA was eluted with water, and the concentration of each sample was determined spectrophotometrically. Finally, the integrity of all RNA samples was monitored on agarose gels.

Total RNA from intact femurs (contains cartilage and marrow), brain, heart, lung, testes, skin, and skeletal muscle was extracted using the ToTALLY RNA kit (Ambion, Austin, TX). Bone was initially pulverized in liquid nitrogen using pestle and mortar before adding to the denaturing solution. All other tissues were added directly to the denaturing solution. Each sample was homogenized with a poltroon (Brinkmann Instruments, Westbury, NY) and extracted once with phenol-chloroform-isoamyl alcohol. The aqueous phase was transferred to a fresh tube, sodium acetate was added, and all samples were extracted again with acid-phenol-chloroform. The resulting aqueous phase was mixed with an equal volume of isopropanol and incubated at −20°C for at least 1 h to precipitate RNA. Finally, the RNA was pelleted, washed with 70% ethanol, air dried, and dissolved in RNase-free water/0.1mM EDTA. Again, concentrations of each RNA sample were determined by spectrophotometry and integrity checked on agarose gels.

Differential mRNA Display

The differential mRNA display method was used as described by Liang and Pardee [1992] using the RNAimage kit (GenHunter Corp., Nashville, TN). Prior to reverse transcription, RNA was treated with DNase I to ensure that no traces of DNA were present. The DNA-free total RNA was then mixed with 1 μM of each of the degenerate oligo-dT-primers (H-T11A, H-T11C, H-T11G, where H is the HindIII site sequence), 1 × reverse transcription buffer, and 20 μM dNTPs. The solution was heated for 5 min at 65°C, then cooled to 37°C for 10 min followed by the addition of 200 U of reverse transcriptase. After incubation at 37°C for 1 h, the mixture was heated for 5 min at 95°C followed by cooling and storage at −20°C. PCR was performed in thin-walled tubes containing 0.2 volumes of reverse transcription reaction, 1 × PCR buffer (10 mM Tris-Cl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), 2 μM dNTPs, ^{33}P-dATP (0.25 μl of 1,200 Ci/mmole), 1 μM of each of the degenerate oligo-dT-primers, 0.2 μM arbitrary primer, and 10 U of AmpliTaq DNA polymerase. Light mineral oil was overlaid in each tube and the PCR reactions were performed as follows: denaturing at 94°C for 30 s, annealing at 40°C for 2 min, and extending at 72°C for 30 s for 40 cycles followed by one cycle of extension at 72°C for 5 min. DNA sequencing loading buffer was added to an aliquot of each reaction and incubated at 80°C for 2 min. Each sample was then loaded onto a 6% denaturing DNA sequencing gel and electrophoresed at 1,700 V. The gel was dried without fixation and exposed directly to Kodak Biomax film overnight at room temperature.

The specific band of interest was excised from the gel, placed in 100 μl water for 10 min, and boiled for 15 min. After a 2-min spin, the supernatant was transferred to a new tube and 10 μl of 3 M sodium acetate, 5 μl of glycogen (10 mg/ml), and 450 μl of 100% ethanol were added. After a 30-min incubation at −80°C, the sample was centrifuged for 10 min at 4°C to pellet the DNA. The pellet was washed with 85% ethanol, air dried, and dissolved in 10 μl water. Four microliters of the sample was used for re-amplification using the corresponding primer set (Arbitrary primer = AAGCTTGACCTTT, Oligo-dT primer = AAGCTTTTTTTTTTTA; AAGCTT in both primers represents a *Hind*III site). Following reamplification, the cDNA fragment was checked on an agarose gel for size consistency and subcloned into the pCR-TRAP vector (GenHunter). The cDNA fragment was subsequently sequenced using the Sequenase Ver. 2.0 DNA Sequencing kit (Amersham).

prtb cDNA Amplification

Primers were designed using the full length *prtb* sequence (GenBank Accession #AF085348) from the BLAST database (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) and the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). A 582 bp cDNA fragment was amplified from a commercial mouse brain cDNA library (Marathon, Clontech, CA) using a primer pair of 5′-CCA-AGGCTCTTCCTTAATTCCTCTG-3′ (*prtb* nt #771-795) and 5′-CTTCTCCCCTGCCTGTCAT-CTTTA-3′ (*prtb* nt #1329-1352) by PCR. This fragment was subcloned using the AdvanTAge PCR cloning kit (Clontech, Palo Alto, CA) and confirmed by DNA sequencing (Sequenase 2.0 DNA sequencing kit (Amersham, Upsala, Sweden). The mouse osteocalcin probe was a kind gift of Dr. Gerard Karsenty, Baylor College of Medicine, Houston, TX), the *c-fos* and *c-jun* probes were purchased from ATCC (Accession #41041 and 63026, respectively).

Northern Blot Analysis

Total RNA (20 μg) from multiple samples was prepared, fractionated on a 1% formaldehyde/agarose gel, transferred to a nylon membrane (Nytran), and UV cross-linked according to standard procedures. cDNA probes were random labeled with ^{32}P-dCTP and hybridized to the membrane at 65°C overnight in a solution containing 15% formamide, 200 mM $NaPO_4$ (pH 7.2), 1 mM EDTA, 7% SDS, and 1% BSA. Following hybridization, the blot was washed in a solution of 2 × SSC/1%SDS at 50°C for 30 min, 0.2 × SSC/1% SDS at 50°C for 30 min, and 0.2 × SSC/0.1%SDS at 65°C for 30 min. Finally, the blot was exposed to Kodak Biomax film at −80°C. The amount of bound probe was quantitated by scanning the X-ray film and measuring the integrated optical density (IOD) of each band using Image-Pro Plus software (Media Cybernetics). The values of bound *prtb* were normalized to the corresponding levels of 18S rRNA (on membrane), and plotted as the ratio of *prtb* to 18S rRNA in arbitrary units.

Bioinformatic Analysis of *prtb* Nucleotide and Amino Acid Sequence

Using the ExPasy molecular biology server (http://www.expasy.ch/) similarity searches using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/), pattern and profile searches using PROSITE (http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html), as well as, specific searches for postranslational modification (PSORTII; http://psort.nibb.ac.jp/ and NetPhos; http://www.cbs.dtu.dk/services/NetPhos/) and transmembrane helices (TMPRED http://www.ch.embnet.org/software/TMPRED_form.html) were performed.

RESULTS

Differential mRNA Display

In order to identify genes that were differentially expressed during early osteoblast adhesion and spreading, we compared mRNA samples from the osteoblastic cell line MC3T3-E1 in suspension with that of cells that were allowed to adhere to a substrate composed of adsorbed serum proteins on tissue culture polystyrene for 30 and 60 min. Microscopically, cells started to adhere 5 min post-plating and spread after 15 min. At 30 min over 90% of the cells showed a flat, spread morphology, had developed focal contacts, and could not be flushed off the surface with several washes of PBS [data not shown; Sommerfeldt et al., 2001].

Using specific arbitrary primers, we identified several cDNA fragments that displayed differential expression during adhesion (Fig. 1). After isolation, cloning, sequencing, and comparison with the available databases, most of the fragments showed no homology or were identified as expressed sequence tags (ESTs) with no known function. Some of the shorter fragments showed partial homology to a large number of previously deposited sequences (data not shown). One cDNA fragment showed a 97% nucleotide homology to mouse *prtb* (Accession #AF085348) and a 96% homology with its human homologue KIAA0058 (Accession # D31767).

Bioinformatic Sequence and Motif Analysis

Both *prtb* and its human homologue KIAA0058 (Accession # D31767) with a 92% sequence identity within the coding region have no assigned function. In order to investigate the possible function of *prtb*, we performed a series of bioinformatic analyses. Motif searches within the predicted 168 amino acid sequence revealed five possible tyrosine phosphorylation sites and a protein kinase C binding site (ProScan and NetPhos). Further, eight myristoylation sites and various enzyme-binding sites were predicted as well as a potential transmembrane helix (TMPRED). PSORT II calculated a likelihood for a cytoplasmic location of 47.8%. In addition, a possibility for a 'paired box' domain was detected using the PROSITE motif search.

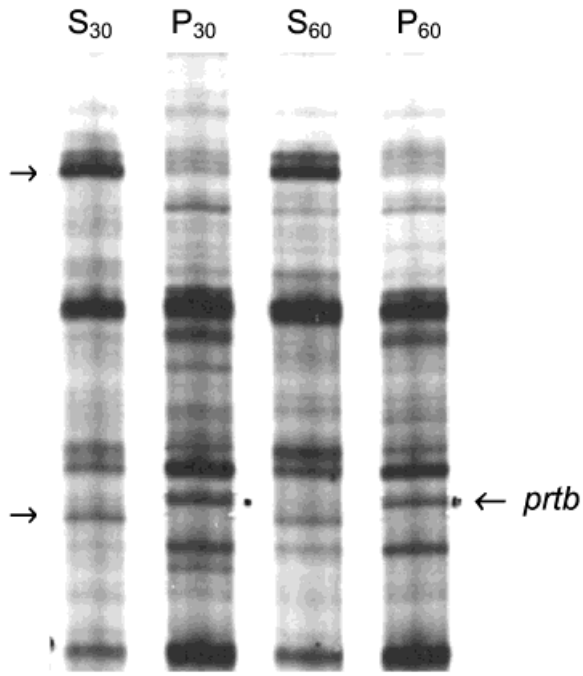

Fig. 1. DD-PCR of adherent vs. suspended MC3T3-E1 osteoblasts. Total RNA was extracted from cell-cycle synchronized, quiescent cells that had either been suspended in serum-free media (S_{30} and S_{60}) or allowed to attach to a serum-protein coated tissue culture polystyrene surface for 30 and 60 min (P_{30} and P_{60}). The arrows on the left indicate two cDNA fragments that appear to be upregulated in suspension. The arrow on the right indicates the *prtb* cDNA fragment, showing upregulation during early osteoblast adhesion and no detectable signal in osteoblasts in suspension.

Expression Analysis of *prtb* mRNA

Northern analysis was performed using a radiolabeled *prtb* cDNA probe hybridized to total RNA from quiescent MC3T3-E1 cells following 30 min of adhesion to either serum-coated or untreated tissue-culture polystyrene (Fig. 2). Similarly, cells in suspension were induced with 10%FBS for 30 min or maintained

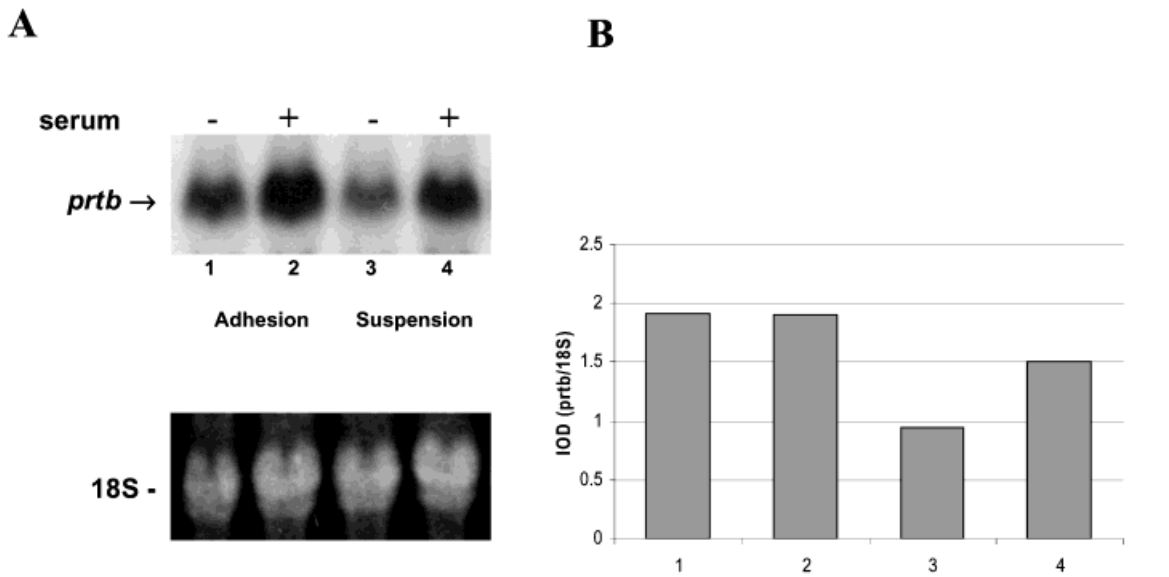

Fig. 2. Northern analysis of serum-induced *prtb* mRNA expression during suspension and adhesion. Cell cycle arrested (G_0), and quiescent cells were allowed to attach to uncoated (**lane 1**) or serum-precoated (**lane 2**) tissue-culture polystyrene for 30 min or kept in suspension in the absence (**lane 3**) or presence (**lane 4**) of serum for the same time as described in Materials and Methods. **A**: Northern analysis using a radiolabeled *prtb* cDNA probe is shown. The lower panel shows the 18S ribosomal RNA on the Nytran membrane used for normalization. **B**: Bar graph indicates the ratio of *prtb* signal on the autoradiography normalized to 18S rRNA on the membrane (from A), as described in Materials and Methods.

in serum-free media for the same time. The detected mRNA had a size of ~1.9 kb, consistent with the 1,844 bp sequence from the database. *Prtb* was expressed in suspended cells at low levels (Fig. 2A, lane 3), but was upregulated two-fold upon adhesion to tissue-culture polystyrene, both in the absence and presence of serum proteins on that surface (Fig. 2A lanes 1 and 2; 2B). In order to determine whether serum proteins could induce *prtb* expression independent of adhesion, serum-starved cells in suspension were stimulated with 10% fetal bovine serum for 30 min. Again, *prtb* expression increased, but only ~1.5-fold, in the stimulated suspended cells (Fig. 2A,B; lanes 3 and 4).

Since *prtb* mRNA was found in the osteoblastic mouse-calvarial cell line MC3T3-E1, we asked the question whether we could also detect expression in bone, as well as other tissues. Therefore, total RNA was isolated from brain, heart, lung, testis, skin, and bone (contains cartilage and marrow) of adult male rats. In this tissue RNA-blot experiment, we confirmed *prtb* expression in brain and bone, and detected weak expression levels in heart, lung, testis, and skeletal muscle of adult rats (Fig. 3). Also, a second mRNA species with an approximate size of 2.4 kb, hybridized to the *prtb* cDNA probe. This band showed the highest level of expression in bone and was absent in the brain (Fig. 3).

prtb mRNA Expression During Osteoblast Differentiation

Next, we asked the question if *prtb* expression is regulated during osteoblast maturation and differentiation (i.e., proliferation, matrix protein synthesis, or matrix mineralization). To this end, MC3T3-E1 cells were used in an in vitro system, in which differentiation was induced by ascorbic acid and β-glycerol phosphate [Quarles et al., 1992]. High expression levels could be detected during the entire time course of differentiation (day 2–31) with no apparent cyclic regulation (Fig. 4). Since osteocalcin expression is known to be highly dependent on osteoblast differentiation, it was used as a positive control for osteoblast differentiation, and as expected, an increase in expression towards the mature phenotype of the osteoblast was detected (Fig. 4).

prtb mRNA Expression During Osteoblast Adhesion

To further elucidate the function of *prtb* during the adhesion of osteoblasts to serum proteins, we investigated its expression during

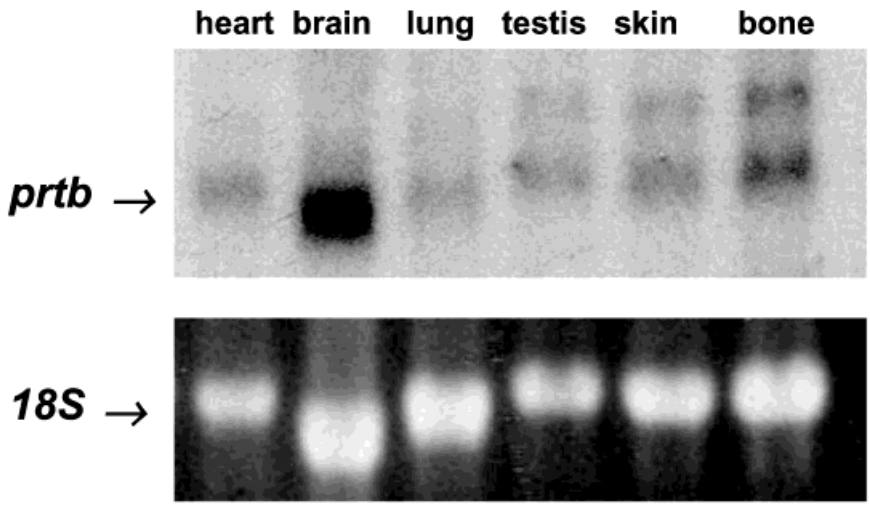

Fig. 3. mRNA expression of *prtb* in various tissues. Total RNA (20 μg) was fractionated on a 1% agarose/formaldehyde gel and Northern analysis was carried out as described in Materials and Methods using a random labeled *prtb* probe. The top panel shows *prtb* expression, the bottom panel indicates the amounts of RNA loaded in each lane via the 18S rRNA signal on the same membrane.

the adhesion process at various early timepoints. Expression was detected at similar levels at all investigated timepoints (5, 10, 15, 20, 25 min) (Fig. 5). This expression pattern was compared with *c-fos* and *c-jun*, two immediate early genes also upregulated during osteoblast adhesion to serum proteins; *c-jun* expression peeked at 15 min and *c-fos* expression at 20 min (Fig. 5). The message for several other genes known to be expressed in osteoblasts during S-phase, such as collagen type I, osteocalcin, alkaline phosphatase, and β1-integrin, were not detected at these very early timepoints (data not shown).

DISCUSSION

Prtb expression had previously been described during embryonic development in various tissues (e.g., heart from E11.5 to E12.5 and throughout the embryo at day E15.5) and in the brain of adult mice [Yang and Mansour, 1999]. Our data offers the first evidence for the expression of this gene in bone. We also observed

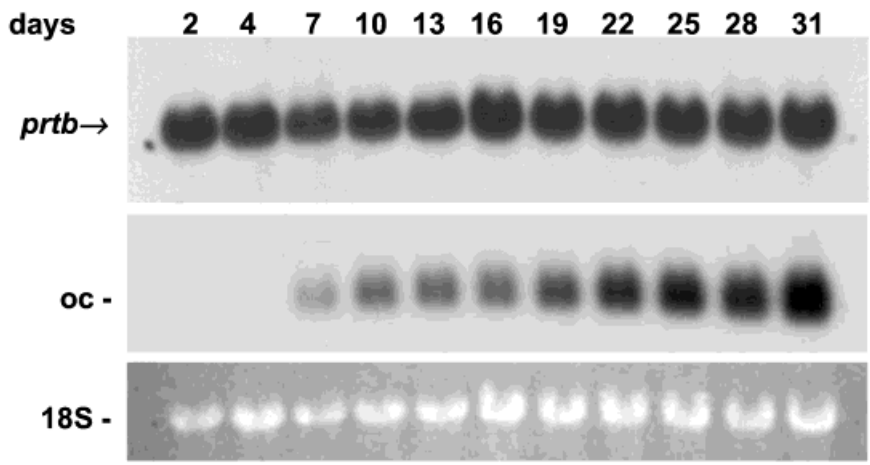

Fig. 4. Expression of *prtb* during differentiation of MC3T3-E1 osteoblasts in vitro. Differentiation of cells from the MC3T3-E1 mouse calvarial osteoblast-like cell line was induced using ascorbic acid and β-glycerol phosphate as described in Materials and Methods. Total RNA was extracted at indicated timepoints (days post-induction), separated on a 1% agarose/formaldehyde gel and Northern blotting was carried out using random labeled cDNA probes for *prtb* (**top panel**) and mouse osteocalcin (**middle panel**). The bottom panel shows the 18S rRNA to indicate the amount of total RNA loaded per lane.

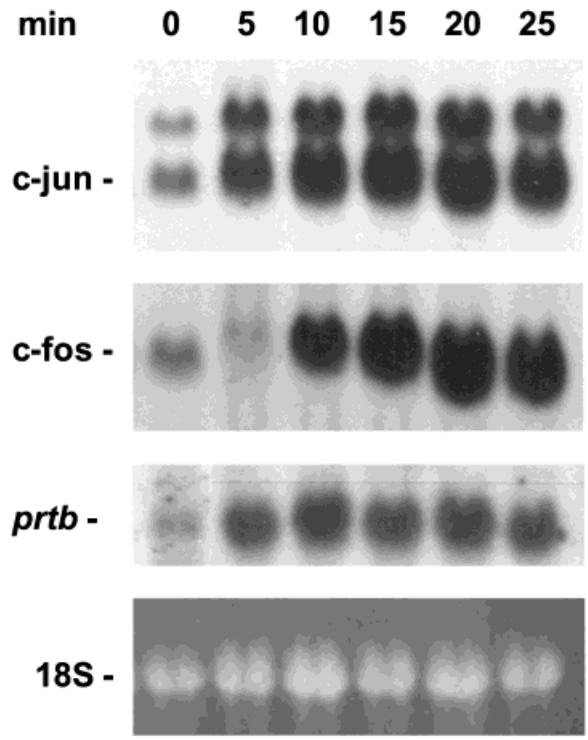

Fig. 5. Expression kinetics of *c-jun, c-fos,* and *prtb* during osteoblast adhesion. Quiescent MC3T3-E1 cells were plated on serum-pretreated tissue culture polystyrene for 5,10, 15, 20, and 25 min as described in Materials and Methods. Cells in suspension served as a control (0). Cells were lysed at the indicated timepoints, mRNA was extracted and fractionated on a 1% agarose/formaldehyde gel. Northern analysis was then performed using random labeled cDNA probes for *c-jun* (**top panel**), c-fos (**second panel from top**), and *prtb* (**third panel**). The bottom panel shows the 18S rRNA to indicate the amount of total RNA loaded per lane.

prtb expression in osteoblasts in vitro, and we were able to show differential regulation of this gene during cell adhesion, cell-matrix interactions, and serum induction.

Although the function of *prtb* remains unclear, an attempt was made to probe the nature of this gene using a transgenic mouse model. In this approach, a gene trap system was used to identify genes expressed in the developing inner ear and a *prtb*-null mutant was generated, which was viable and fertile. No functional abnormalities were detected either in inner ear or brain, as well as other tissues examined [Yang and Mansour, 1999]. Despite an unclear functional role of *prtb* using the null mice approach, computational data (from the various databases and motif searching algorithms) point towards a role for *prtb* during signal transduction, since post-translational modification via tyrosine phosphorylation, a predominantly cytoplasmic localization, and a transmembrane domain were identified.

Upregulation of *prtb* mRNA levels as early as 5 min post-plating of cell cycle synchronized quiescent cells is suggestive for a possible function during very early cell adhesion. In this respect, similar experiments have been conducted in other cell types such as capillary endothelial cells, fibroblasts, and T lymphocytes, including analysis of expression of well-known immediate early cell cycle regulatory genes (e.g., *c-fos*, *c-jun*, and *c-myc*). It is evident from these and other studies that adhesion to extracellular matrix proteins induces re-entry of quiescent cells into the cell cycle with consecutive upregulation of several immediate early genes regulating cell cycle progression and proliferation [Boudreau et al., 1995; Dike and Ingber, 1996; Troussard et al., 1999; Bianchi et al., 2000].

Since we showed that *prtb* expression is upregulated during adhesion of quiescent cells to a substrate with very similar kinetics to *c-fos* and *c-jun*, one can speculate about a possible role for *prtb* during processes usually associated with these molecules. The processes include transcriptional activation, cell cycle re-entry, and control of proliferation. This 'guilt-by-association' approach is becoming increasingly popular in cluster analyses within genome-wide studies, where molecules are linked functionally due to similarities in their expression pattern kinetics [Lockhart and Winzeler, 2000]. The possibility of a 'paired box' domain and a transmembrane helix within *prtb* is interesting in this context. Although lacking a DNA binding site on its own, *prtb* could serve as a transcriptional coactivator binding to immediate early genes containing a 'paired box' domain [Kioussi and Gruss, 1994]. As mentioned previously, *prtb* localization within the cell by computer simulation was hypothesized to be most likely in the cytoplasm, which would be inconsistent with a role as a transcriptional coactivator. Nevertheless, *c-jun*, although predominantly found in the cytoplasm, also travels into the nucleus upon activation.

Considering the possible transmembrane domain of *prtb* and its upregulation during adhesion per se, even a shuttle function from the membrane, through the cytoplasm, and into the nucleus, similar to the one recently described for the transcriptional coactivator JAB1, can be imagined [Bianchi et al., 2000]. In this context,

our data showing serum induction of *prtb* slightly higher in adherent cells vs. cells in suspension would be consistent with the notion of *prtb* expression being primarily controlled by the presence of serum proteins but amplified when cells are allowed to adhere to a substrate.

Finally, the predicted tyrosine phosphorylation and N-myristoylation sites are also consistent with a signaling function of *prtb*, since post-translational modification is a common feature among proteins involved in signal transduction [Taniguchi, 1999].

In summary, we show that *prtb* is differentially expressed in suspended and adherent osteoblast-like cells, and that it is upregulated in the presence of serum proteins. Its expression in bone cells was confirmed on the tissue level via Northern blots, which also showed expression in the brain and long bones of adult rats. In these tissue blots, a second and larger than the known 1.9 kb mRNA species expressed highly in the brain, was detected with highest levels in bone. These results suggest that the *prtb* gene can be alternatively spliced, but can also be interpreted as evidence for other genes from a larger family with partial homology to *prtb*. Lastly, protein binding assays to determine binding partners for *prtb*, in situ hybridization studies, and examination of bone and osteoblasts isolated from *prtb* knock-out mice, will help to further characterize the function of this interesting gene in the skeletal system.

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