The role of annexin 2 in osteoblastic mineralization

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Summary

While the basic cellular contributions to bone differentiation and mineralization are widely accepted, the regulation of these processes at the intracellular level remains inadequately understood. Our laboratory recently identified annexin 2 as a protein involved in osteoblastic mineralization. Annexin 2 was overexpressed twofold in SaOSLM2 osteoblastic cells as a fusion protein with green fluorescent protein. The overexpression of annexin 2 led to an increase in alkaline phosphatase activity as well as an increase in mineralization. Our data suggest that the increase in alkaline phosphatase activity does not result from increased alkaline phosphatase transcript or protein levels; therefore we evaluated mechanism of action. We determined that both annexin 2 and alkaline phosphatase activity were localized to membrane microdomains called lipid rafts in osteoblastic cells. Annexin 2 overexpression resulted in an increase in alkaline phosphatase activity that was associated with lipid microdomains in a cholesterol-dependent manner. Furthermore, disruption of lipid rafts with a cholesterol sequestering agent or reduction of annexin 2 expression by specific antisense oligonucleotides each resulted in diminished mineralization. Therefore, intact lipid rafts containing annexin 2 appear to be important for alkaline phosphatase activity and may facilitate the osteoblastic mineralization process.

Key words: Lipid rafts, Bone, Alkaline phosphatase, Annexin 2, Osteoblast

Introduction

Bone is a dynamic tissue that is replaced throughout life. The normal process of bone turnover requires several levels of communication between the osteoblasts, which produce bone, and the osteoclasts, which resorb it. Osteoblasts have the primary function of making and secreting osteoid, the extracellular matrix (ECM) and associated proteins required for mineralization. A variety of proteins have been implicated in the bone mineralization process including collagen I and alkaline phosphatase (ALP). Collagen I provides the structural framework for bone formation and mineral deposition, and the collagen fibers give tensile strength to bone. ALP catalyzes the hydrolysis of phosphomonoesters at an alkaline pH and breaks down pyrophosphate, which is an important inhibitor of calcium phosphate deposition at the extracellular level (Johnson et al., 2000; Miao and Scutt, 2002). Four isozymes of ALP have been identified in humans: tissue non-specific liver/bone/kidney, intestinal, placental, and germ cell ALP (Miao and Scutt, 2002). Regulation of the localization and activity of ALP and other proteins is important in the ultimate control of bone differentiation and mineralization.

Recently, we isolated annexin 2 (anx2) in a screen for differentially expressed genes in bone malignancy. Data from our laboratory suggest that anx2 is not affecting growth of cells (a hallmark of cancer), but may be facilitating osteoblastic mineralization (unpublished results). Historically, anx2 was identified as a src-kinase substrate and belongs to a large family of structurally related proteins (Erickson and Erickson, 1980; Radke et al., 1980). Annexins are characterized by conserved annexin repeat domains and an ability to bind negatively charged phospholipids in a calcium-dependent manner. Although the physiological roles of the annexins require further clarification, they have been described as participants in processes such as endo- and exocytosis, membrane fusion, membrane/cytoskeletal interactions, and voltage-dependent calcium channels (Creutz, 1992; Drust and Creutz, 1988; Faure et al., 2002; Harder et al., 1997; van de Graaf et al., 2003). Anx2 has been described as a cell surface receptor for ECM molecules such as tenascin-C and proteolytic enzymes including tissue plasminogen activator and cathepsin B (Chung and Erickson, 1994; Fitzpatrick et al., 2000; Mai et al., 2000; Siever and Erickson, 1997).

Anx2 has also been implicated in the organization and dynamics of membrane rafts through binding to actin and lipids in the plasma membrane (Gerke and Moss, 2002). Anx2 has been isolated from lipid microdomain-associated protein complexes that include molecules such as CD44, actin and cytoskeletal interacting proteins including members of the ezrin/radixin/moesin family (Oliferenko et al., 1999). Although the role of anx2 in such complexes remains unclear, there is evidence to suggest that it facilitates the aggregation of microdomains in smooth muscle cells during contraction or the adherence of enteropathogenic bacteria to the cell surface (Babiychuk and Draeger, 2000; Zobiack et al., 2002). In osteoblastic cells, we have shown that anx2 is not localized to the nucleus or the cell surface; rather it is restricted to the cytoplasm and the intracellular aspect of the plasma membrane where it may interact with lipid microdomains (unpublished results).

A growing body of evidence suggests that cholesterol-rich

membrane microdomains are present in the membranes of many if not all mammalian cells. Lipid microdomains or 'lipid rafts' are small (10-300 nm) membrane regions that are transient in nature and highly dynamic (Pierini and Maxfield, 2001). More than 100 proteins including glycosylphosphatidylinositol (GPI)-anchored proteins have been suggested to be associated with lipid rafts (Abrami et al., 2001). ALP is a GPI-anchored protein and therefore is predicted to localize to the extracellular aspect of lipid rafts. In fact, placental ALP has been shown to associate with lipid rafts in vitro (Saslowsky et al., 2002). Proteins that localize to the cytoplasmic face of lipid rafts include members of the src-family kinases, protein kinase C and actin (Simons and Ikonen, 1997; Uittenbogaard et al., 2002). Functionally lipid rafts are believed to form recruitment platforms for proteins involved in signaling cascades, and have also been implicated in the sorting of lipids and proteins in the secretory and endocytic pathways (Cherukuri et al., 2001; Galbiati et al., 2001).

In the present study, we evaluated the effects of anx2 overexpression in osteoblastic cells. Our data demonstrate that anx2 overexpression results in enhanced mineralization and increased ALP activity. Osteoblastic lipid rafts were isolated and both anx2 protein and ALP activity were detected. Overexpression of anx2 resulted in an increase in ALP activity specifically at these microdomains. Both disruption of lipid rafts and reduced expression of anx2 diminished mineralization. Together these studies suggest that lipid raft integrity and the presence of anx2 at these domains may be important for mediating osteoblastic mineralization.

Materials and Methods

General materials and reagents

All chemicals were obtained from Sigma (St. Louis, MO) and cell culture plastics were obtained from Midwest Scientific (Valley Park, MO) unless otherwise stated.

Human cell culture

The human osteosarcoma cell line SaOSLM2 was kindly provided by Robert Radinsky, Ph.D., University of Texas MD Anderson Cancer Center (Radinsky et al., 1994). Normal human osteoblasts (hOST) were obtained from BioWhittaker, Walkersville, MD. All cells were grown in RPMI 1640 supplemented with 10% FBS. Mineralized nodule formation was induced by culturing the cells in Osteoblast Growth Medium or αMEM supplemented with 200 nM hydrocortisone 21hemisuccinate, 50 μg/μl ascorbic acid and 7.5 mM β-glycerophosphate (Biowhittaker).

Overexpression of anx2 in osteoblastic cells

The full-length anx2 gene (Incyte Genomics, Palo Alto, CA) was subcloned into the pEGFP vector (Clontech, Palo Alto, CA) and verified by sequence analysis. The green fluorescent protein (GFP) tag was cloned 3′ of the anx2 gene, which resulted in a fusion protein with the GFP tag at the carboxyl terminus of anx2 (anx2GFP). SaOSLM2 cells were stably transfected with the anx2GFP fusion construct using the calcium phosphate transfection technique and G418 selection (Graham and van der Eb, 1973).

Membrane isolation

Cells were swollen in intracellular buffer (ICB) (20 mM Hepes, 120 mM KCl, 12 mM NaCl, 1.62 mM $MgSO_4$, 1 mM EDTA, 0.5 mM $CaCl_2$) supplemented with 0.25 M sucrose, and gently disrupted using a Dounce tissue grinder (Kontes, Vineland, NJ). During the course of cell disruption, cell integrity was monitored by microscopy. Nuclei were pelleted by low speed centrifugation (800 $\mathbf{g}$ for 10 minutes). The post-nuclear supernatant was then subjected to high-speed centrifugation (100,000 $\mathbf{g}$ for 1 hour) to pellet insoluble membranes. For experiments involving digitonin treatment, the membrane fraction was resuspended in ICB containing 0.01% digitonin solubilized in ethanol for 10 minutes on ice. Following treatment, the membrane fractions were brought to 5 ml with ICB and centrifuged at 100,000 $\mathbf{g}$ for 1 hour. The supernatants (wash fractions) were removed and concentrated to 200 μl using Centricon filter devices (Millipore Corporation, Before, MA) followed by immunoblot analysis. The pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% Ipegal, 0.1% SDS, 0.5% deoxycholic acid) and were then used in immunoblot analyses.

Immunoblot analysis

Protein aliquots were quantitated with the BCA system (Pierce, Rockford, IL) and electrophoresed through 12% SDS polyacrylamide gels. Gels were electrotransferred to PVDF membranes (Scheicher & Schuell, Inc., Keene, NH) using the mini transblotter system (Bio-Rad Laboratories, Hercules, CA). The PVDF membranes were blocked in 5% nonfat dry milk containing 0.2% Tween 20 in PBS. Primary anx2 antibodies (Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) were diluted 1:1000 in blocking reagent and membranes were incubated at 4°C overnight. Membranes were washed in PBS containing 0.2% Tween 20 for 3× 20 minutes. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA or Amersham Biosciences, Piscataway, NJ) was diluted 1:1000 in blocking reagent and incubated on the PVDF membrane for 2 hours at ambient temperature. The membranes were washed as above and visualized using chemiluminescence according to the manufacturer's protocol (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

Immunofluorescence and confocal microscopy

Cells were cultured overnight on chambered slides (Nunc, Inc., Naperville, IL). Adherent cells were rinsed with PBS and fixed in 3.7% formaldehyde, 0.12 M sucrose in PBS for 10 minutes. The fixed cells were rinsed with PBS, permeabilized with methanol at –15°C for 15 minutes, and blocked in 15% donkey serum (Jackson Immuno Research, West Grove, PA) in PBS. ALP primary antibody (DSHB, Iowa City, IA) was diluted 1:1000 and incubated overnight at ambient temperature in a humidified Petri plate. Cells were blocked with 15% donkey serum in PBS and incubated with diluted rhodamine-conjugated donkey anti-mouse antibody (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for at least 1 hour at ambient temperature. Cells were washed with PBS, mounted with Vectashield (Vector Laboratories, Burlingame, CA) and visualized either with a TE 200 Nikon inverted microscope with fluorescence or a Nikon PCM 2000 confocal microscope.

RT-PCR

Total cytoplasmic RNA was isolated using TRIZOL® reagent according to the manufacturer's instructions (Life Technologies, Rockville, MD). RNA was then subjected to first strand cDNA synthesis using the Superscript cDNA synthesis kit (Life Technologies, Carlsbad, CA). First strand cDNA was amplified using sequence-specific primers (Life Technologies). (ALP forward, ACGTGGCTAAGAATGTCATC. ALP reverse, CTGGTAGGCGAT-GTCCTTA. G3 forward, TGCCAAGGCTGTGGGCAAGG. G3 reverse, GCTTCACCACCTTCTTGATG.) Fifty microliter PCR reactions containing 0.5 μg-1.0 μg of first strand cDNA were initiated with an annealing temperature of 56°C and 10 μl aliquots were

removed at 25, 30 and 35 cycles. PCR products were visualized on agarose gels with ethidium bromide to provide a semi-quantitative analysis of specific mRNA expression.

Detection of calcium nodules

Cells were maintained in either RPMI or in medium to induce differentiation. In order to detect insoluble calcium nodules, the cell monolayer was rinsed with PBS and fixed with 100% cold methanol for 10 minutes at –20°C. The methanol was removed with two washes of deionized water and insoluble calcium was stained with Alizarin Red S as previously described (Jensh and Brent, 1966). Residual stain was removed with 0.1% sodium acetate, pH 6.4 and the presence of nodules was documented by bright-field microscopy.

Alkaline phosphatase activity assay

ALP activity was measured in cell monolayers using a *p*-nitrophenyl phosphate substrate kit (Sigma-Aldrich, Saint Louis, MI). Activity was detected at 405 nm in a microplate reader. One unit of alkaline phosphatase hydrolyzes 1 μmol *p*-nitrophenol in ten minutes.

Lipid raft isolation

Lipid rafts were isolated from osteoblastic cells by Triton X-100 disruption followed by high-speed centrifugation. SaOSLM2 and SaOSLM2-anx2GFP cells were lysed in ICB containing 1% Triton X-100 and used to generate a linear gradient of 5-40% sucrose that was subjected to high-speed centrifugation (180,000 *g* for 6 hours, 4°C). Fractions (500 μl) were sequentially removed from the top of the gradient. Each of the nine fractions was diluted to 5 ml in ICB reducing the sucrose concentrations to between 0.5 and 4.0%. The fractions were centrifuged to pellet the insoluble raft complexes from the soluble supernatant (100,000 *g* for 1 hour, 4°C). The insoluble pellet was disrupted with RIPA and the supernatant was concentrated for use in immunoblot analyses and ALP activity assays.

Cholesterol disruption of cells in culture

Cells were cultured in 0.5 mM methyl-β-cyclodextrin in conditions to induce differentiation. Mineralization was evaluated on day 5 by Alizarin Red S staining. Stain was solubilized in 10% cetylpyridinium chloride for 1 hour at room temperature and quantitated at 570 nm.

Anx2-specific morpholino antisense oligonucleotide treatment of osteoblastic cells

Confluent monolayers of SaOSLM2 cells were treated with an anx2-specific or a non-specific morpholino oligonucleotide for 5 hours in serum-free medium following the protocol suggested by the manufacturer (Gene Tools, LLC, Philomath, OR). After 5 hours, the medium was replaced with differentiation-inducing medium for 4 days. Cells were then lysed for immunoblot analysis or stained with Alizarin Red S to detect mineralization.

Results
Overexpression of anx2 in osteoblastic cells

In order to determine the potential role of anx2 in osteoblastic cells, we developed an anx2GFP fusion construct that was stably transfected into SaOSLM2 osteoblastic cells. Anx2 was overexpressed by approximately two- to threefold in the osteoblast-like cells (Fig. 1A). Visualizing the anx2GFP fusion protein by epifluorescence microscopy revealed nuclear exclusion as was previously characterized for endogenous anx2 in osteoblastic cells (Fig. 1B) (unpublished results).
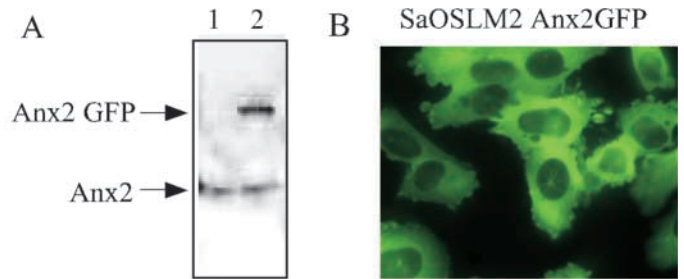
Fig. 1. Anx2 overexpression in osteoblastic cells. SaOSLM2 cells were stably transfected with GFP or an anx2GFP fusion construct. (A) Immunoblot analysis confirmed that endogenous anx2 was expressed similarly in the GFP (1) and the anx2GFP (2) transfectants and the exogenous fusion protein was expressed in the transfectants. Endogenous anx2 was detected at approximately 36 kDa whereas the fusion protein was shifted to approximately 63 kDa because of the 27 kDa GFP tag. (B) Epifluorescence microscopy of GFP reveals that the fusion protein is excluded from the nucleus, similarly to endogenous anx2 protein expression (unpublished results) (200×).

Subcellular fractionation revealed a similar cytoplasmic and membrane profile for endogenous and exogenous anx2. Therefore, we initiated studies to evaluate the phenotypic affects of anx2 overexpression.

Anx2 overexpression enhances mineralization of osteoblastic cells

We originally identified anx2 in a differential expression screen for bone malignancy (unpublished results). Having determined that anx2 overexpression did not affect cell growth under normal culture conditions, we evaluated whether anx2 was involved in the differentiation process. In order to determine whether anx2 expression had an effect on bone differentiation, we cultured the cells in the presence of ascorbic acid and β-glycerophosphate. After the differentiation period, the formation of calcium nodules characteristic of osteoblastic mineralization were detected by Alizarin Red S staining. As illustrated in Fig. 2, differentiation-induced calcium nodules were detected in normal osteoblasts, SaOSLM2GFP cells, and in the anx2GFP-expressing cells. An increase in nodule formation was observed upon anx2 overexpression. Therefore, we suggest that anx2 functions to facilitate mineralization, a terminal step in the differentiation process of osteoblastic cells.

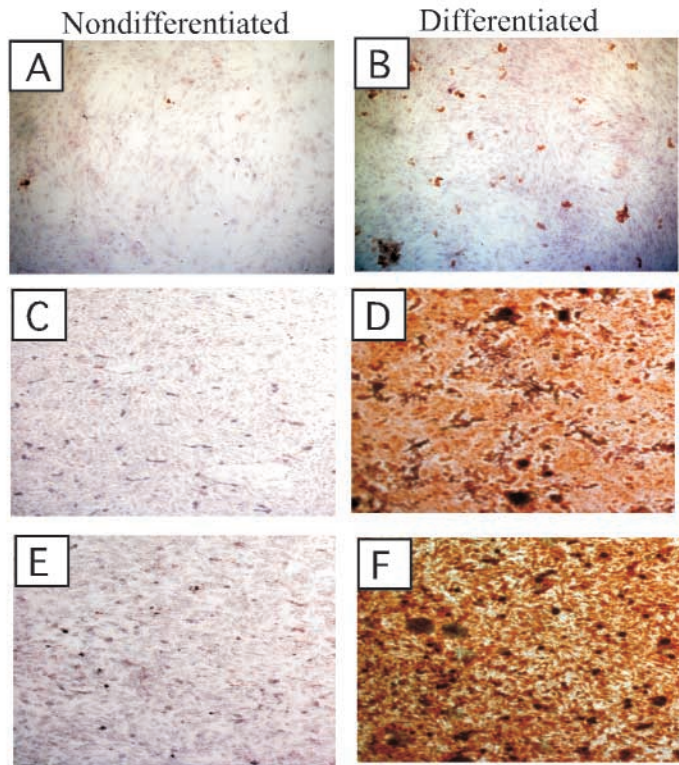### Anx2 overexpression results in an increase in ALP activity

One mechanism by which anx2 may be enhancing osteoblastic mineralization is through an induction in ALP activity. Immunofluoresence in anx2-overexpressing cells illustrated a colocalization of anx2 with ALP at the periphery of the cell (Fig. 3A). To determine whether anx2 expression had an effect on ALP activity, cells were cultured under normal and differentiation conditions (Fig. 3B). Anx2 overexpression increased the basal level (normal culture conditions) of ALP activity as compared to that in SaOSLM2GFP cells. Following differentiation, SaOSLM2GFP cells displayed an expected increase in ALP activity, while cells expressing anx2 had further enhanced ALP activity relative to GFP-containing cells.

These data suggest that overexpression of anx2 leads to an increase in ALP activity that is further elevated following differentiation.

Anx2 may alter ALP activity and thus induce mineralization either by increasing the production of ALP or by enhancing enzymatic activity. To evaluate changes in ALP mRNA expression, semi-quantitative RT-PCR analysis was performed with RNA isolated from parental and anx2-overexpressing cells. No significant changes in ALP mRNA levels were detected between parental and anx2-overexpressing cells under normal culture conditions (Fig. 4) or during differentiation (data not shown). Immunoblot analysis also detected no change in ALP protein levels with anx2 overexpression (data not shown). We therefore suggest that anx2 may be altering ALP enzyme activity. ALP is an extracellular protein (Jemmerson and Low, 1987) whereas anx2 is intracellular (unpublished results), therefore we evaluated whether both proteins resided in a common structural domain in the membrane that may enable intermolecular crosstalk.


Fig. 2. Anx2 overexpression enhances osteoblastic mineralization. A panel of cell cultures were maintained under normal conditions (A,C,E) or under conditions to induce differentiation (B,D,F). All cells were then stained with Alizarin Red S to detect insoluble calcium nodules and analyzed at 40× magnification.

Anx2 fractionates to a Triton X-100 cellular component in a cholesterol-dependent manner

Biochemical analysis of anx2 localization detected a TritonX-100 soluble and an insoluble fraction (Fig. 5A), however anx2 was not detected in the soluble conditioned medium of GFP-expressing or anx2-overexpressing cells (Fig. 5B). The Triton X-100 soluble form of anx2 appears to represent the monomeric (cytoplasmic) pool of protein as determined by sucrose gradient or gel filtration chromatography (data not shown). The insoluble fraction may be consistent with protein localization at membrane microstructures called lipid rafts. Lipid rafts, which are Triton X-100 resistant, are frequently stabilized by cholesterol. To determine whether anx2 associates with membranes in a cholesterol-dependent manner, membrane fractions were isolated from SaOSLM2 cells and treated with digitonin (a cholesterol sequestering agent). The sequestration of cholesterol by digitonin resulted in the release of anx2 from the membrane fraction and the detection of anx2 in the wash fraction (Fig. 5C). These studies suggest that
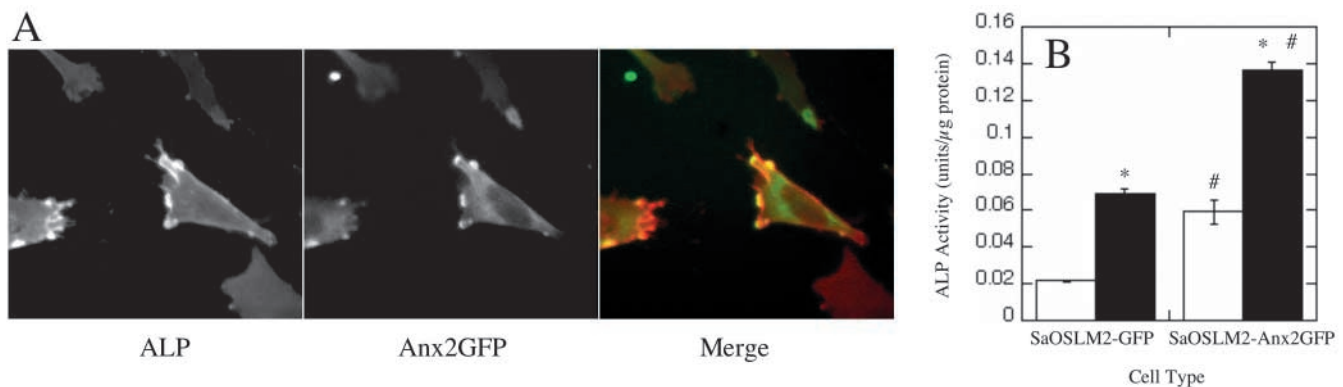
Fig. 3. Anx2 overexpression results in an increase in ALP activity. (A) ALP protein was detected by immunofluorescence in SaOSLM2 cells overexpressing anx2. (B) Cell-associated ALP activity was measured in osteoblast-like cells cultured in both non-differentiation- (white bars) and differentiation-inducing (black bars) media. * Statistically significant increases in ALP activity by culturing the cells under differentiation conditions ($P<0.05$). #Statistically significant increases in ALP activity induced by anx2 overexpression ($P<0.05$).
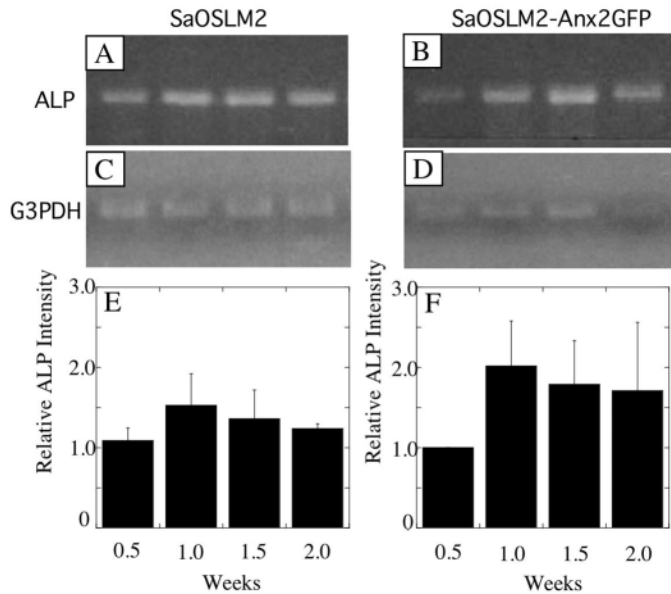
Fig. 4. Anx2 overexpression does not alter ALP gene expression levels. (A) 1 μg of total RNA from SaOSLM2 (A) or SaOSLM2-Anx2GFP (B) cells was subjected to semi-quantitative RT-PCR analysis of ALP or G3PDH message levels. RNA was isolated from cells plated for 0.5, 1.0, 1.5 and 2.0 weeks. Band intensities for ALP signal from GFP- (C) and anx2GFP- (D) expressing cells were normalized against those obtained for G3PDH in the same samples (E,F).

membrane-associated anx2 is at least in part mediated by cholesterol.

Anx2 localizes to lipid rafts

Anx2 associates with membranes that are resistant to Triton X-100 solubilization in a cholesterol-dependent manner suggesting localization to lipid rafts. To explore a lipid raft association, we isolated membrane rafts by subjecting the Triton X-100-resistant cellular components from SaOSLM2 and SaOSLM2-anx2GFP cells to sucrose gradient centrifugation. Immunoblot analysis was conducted with proteins isolated from each fraction across the gradient. Anx2 was detected in fractions four to seven near the center of the gradient (Fig. 6A), consistent with proteins that float because of interactions with buoyant lipid structures.

The activity of ALP in lipid rafts is positively affected by anx2 overexpression

A variety of proteins have been identified as being associated with lipid rafts including GPI-anchored proteins. Placental ALP is a protein previously shown to be localized to lipid rafts via the GPI-anchor (Jemmerson and Low, 1987). To determine whether tissue non-specific ALP is also localized to these microdomains, raft fractions were isolated from SaOSLM2 cells and analyzed for ALP activity. ALP activity was detected in fractions four to seven across a density gradient suggesting an interaction with buoyant lipids (Fig. 6B, solid line). These data demonstrate that anx2 and ALP are both localized to lipid rafts in osteoblastic cells. In addition, we evaluated the activity
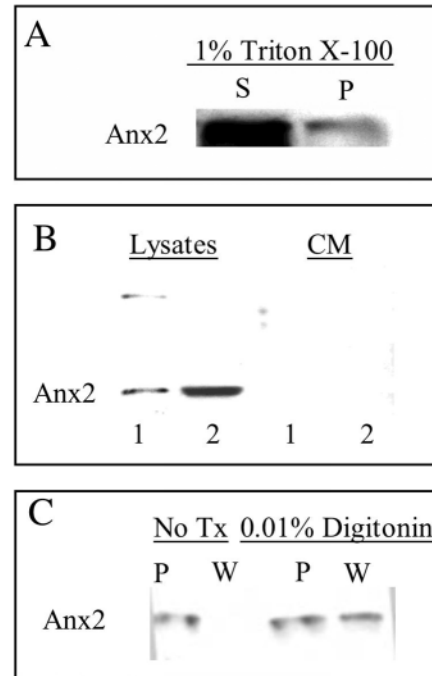

Fig. 5. Osteoblastic cells contain detergent insoluble anx2. (A) Solublization of U2OS cells in 1% Triton X-100 results in a soluble fraction of anx2 (S), and an insoluble fraction of anx2 (P). (B) Cellular lysates were made from both SaOSLM2-anx2GFP- (1) and GFP- (2) expressing cells. Conditioned medium (CM) was also collected from these cells. (C) The membranous fraction following subcellular fractionation of SaOSLM2 cells was treated with 0.01% digitonin or the diluent methanol for 10 minutes. Membranes were resuspended and these insoluble membrane pellets (P) as well as the soluble wash (W) components were analyzed by immunoblot for the presence of anx2.

of lipid raft-associated ALP in anx2-overexpressing cells. We detected a threefold increase in ALP activity associated with lipid rafts isolated from anx2-overexpressing cells as compared to those isolated from parental cells (Fig. 6B, dashed line). Therefore, the observed increase in ALP activity upon anx2 overexpression seen in Fig. 2 appears to occur, at least partially, at lipid rafts. In addition, treatment of lysates with digitonin resulted in loss of detection of anx2 protein and ALP activity associated with lipid rafts (data not shown). Therefore, formation or maintenance of these membrane microstructures, perhaps through stabilization by a scaffolding activity of anx2, may mediate the mineralization process, at least in part by affecting ALP activity.

Lipid rafts and anx2 are both necessary for osteoblastic mineralization

Our data demonstrate that lipid rafts can be isolated from osteoblastic cells and the rafts contain anx2 and ALP. In the literature, lipid rafts have been implicated in a variety of cellular processes, and we would suggest that lipid rafts sequester the necessary components in a temporal and spatial manner to facilitate osteoblastic mineralization. To evaluate more directly whether lipid microdomains are involved in mineralization, osteoblastic cells were treated with a
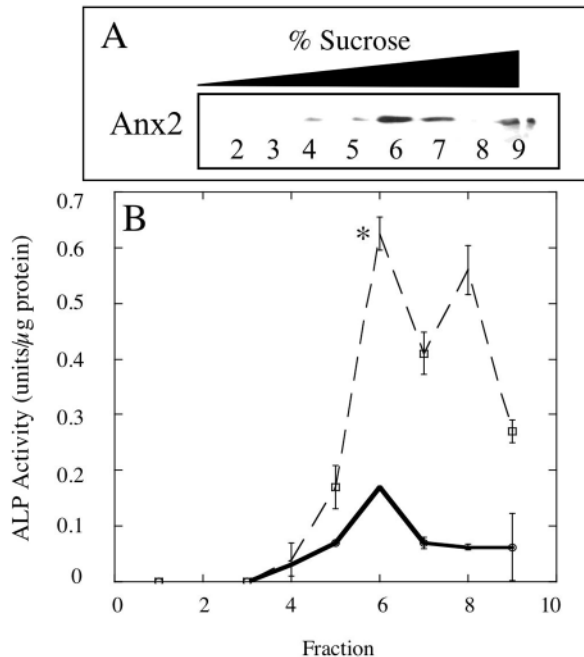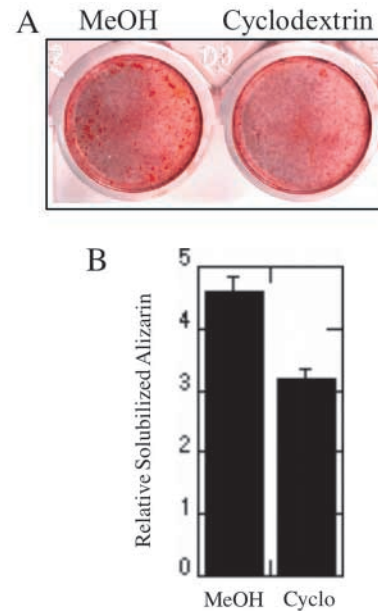
Fig. 6. Anx2 and ALP are associated with lipid rafts. (A) Lipid raft microdomains were isolated from SaOSLM2 and SaOSLM2-anx2GFP cells by centrifugation through a 5-40% sucrose gradient. Fractions were collected and insoluble material was pelleted from each fraction in which the sucrose had been reduced. Anx2 was detected by immunoblot analysis. (B) ALP activity was evaluated in the lipid raft fraction pellets isolated from SaOSLM2 (solid line) and SaOSLM2-anx2GFP (dashed line) cells. The * in fraction 6 represents statistical significance between anx2-overexpressing ALP activities and parental ALP activities ($P<0.05$).

Fig. 7. Cells cultured in the presence of a cholesterol-sequestering agent have a reduced ability to mineralize. SaOSLM2 cells (A) were cultured in conditions to induce mineralization in the presence or absence of 0.5 mM methyl-β-cyclodextrin and stained with Alizarin Red S. The Alizarin Red S was solubilized in 10% cetylpyridinium chloride and quantitated by absorbance at 575 nm (B). OD values were normalized to that obtained from cells maintained in normal conditions.

cholesterol-sequestering agent and examined for their ability to produce mineral crystals. Fig. 7 illustrates that disruption of lipid rafts by culturing cells in the presence of methyl-β-cyclodextrin diminishes mineralization. These effects are not due to cytotoxicity because the presence of methyl-β-cyclodextrin (up to 1 mM) does not affect proliferation of cells in culture (data not shown). These data suggest that cholesterol-mediated lipid rafts may be necessary for osteoblastic mineralization.

In addition to lipid rafts, we wanted to evaluate the necessity for anx2 in the process of mineralization. Fig. 8A demonstrates the use of sequence-specific morpholino antisense oligonucleotides to obtain a greater than 2-fold reduction in anx2 protein levels in SaOSLM2 cells (when normalized to actin expression). The morpholino-treated cultures were maintained in differentiation conditions to determine effects on mineralization. Concomitant with a reduction in anx2 expression was a reduced level of mineralization (Fig. 8B,C) as well as a slight reduction in ALP activity (Fig. 8D). These data suggest that anx2 expression and the integrity of lipid raft structures are both important for osteoblastic mineralization.

Discussion

During endochondral ossification, chondrocytes undergo a series of differentiation events that culminate with the release of matrix vesicles (MV) at the terminally differentiated stage.

These MV contain ALP, anx5 and anx2, and serve to initiate mineralization of the ECM (Anderson, 1995). Anx2 has been suggested to function similarly to anx5, which is described as a potential calcium channel (Anderson, 1995). Annexin channels have yet to be confirmed in vivo, and additional studies indicate that the anx2 heterotetrameric complex may not serve directly as a channel, but rather function to regulate channels (Gerke and Moss, 2002; van de Graaf et al., 2003). Anx5 knockout mice have normal cartilage and bone and the authors suggest that other annexins may be providing compensatory functions (Brachvogel et al., 2003). It is possible that other annexins may compensate for anx2 in osteoblastic cells. In primary cultures of human osteoblasts, annexins 1, 2, 4, 5 and 6 were detected (Mohiti et al., 1995). Based on functional and structural characteristics, anx1 and 5 would be the most likely candidates for compensatory function. Further studies are required to evaluate these possibilities.

Interestingly, upregulation of anx2 has been correlated with the joint destruction and pathologic mineralization associated with both rheumatoid and osteoarthritis (Justen et al., 2000; Kirsch et al., 2000). It has been suggested that the progressive breakdown observed in osteoarthritic cartilage may be the result of MV release, enzyme upregulation and abnormal calcification in the joints (Einhorn et al., 1985). Anx2 has been shown to induce chondrocyte mineralization in culture (Wang and Kirsch, 2002); however, our studies are the first to implicate anx2 in the mineralization process of osteoblastic cells.

MV have also been described in the mineralization of woven bone, but whether MV are involved in the
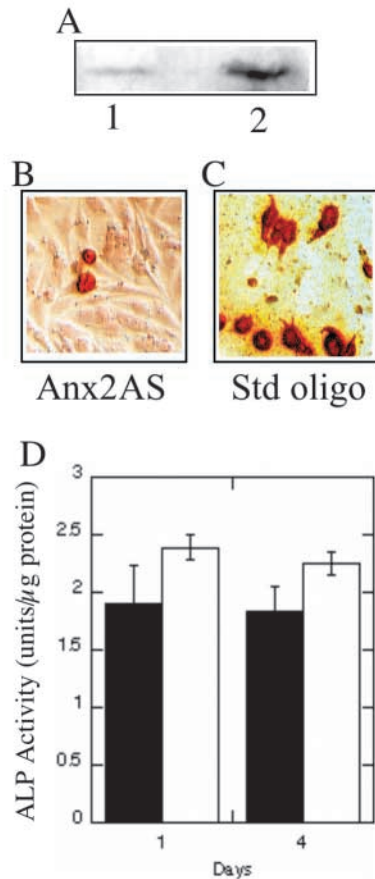
Fig. 8. Reduced anx2 expression in SaOSLM2 cells diminishes alkaline phosphatase activity and mineral crystal formation. (A) SaOSLM2 cells were transfected with an anx2-specific antisense oligonucleotide (1) or a control oligonucleotide (2). (B,C) Anx2 expression was analyzed by western blot at day 4. SaOSLM2 cells with either the anx2-specific oligonucleotide (Anx2AS) or the standard oligonucleotide (Std) were exposed to conditions to induce mineralization for 4 days. The cultures were stained with Alizarin Red S to detect deposition of mineral crystal (200×). (D) ALP activities were also measured in the Anx2AS (black) and the Std (white) oligonucleotide-treated cells.

mineralization of more mature forms of bone remains unclear (Bernard, 1978). The mechanism by which mature bone mineralizes is not as clearly defined. The process of bone mineralization is complicated and requires a tightly regulated production of a variety of proteins including ALP. In fact ALP has been described clinically as the most relevant enzyme in the diagnosis of bone diseases (Hoof and Bore, 1994; Miao and Scutt, 2002). Certain genetic mutations in the ALP gene result in the disease state known as hypophosphatasia. This inherited disorder is characterized by defective bone mineralization and deficiency of serum and tissue non-specific ALP activity (Mornet, 2000). These patients present with varying degrees of bone maintenance and repair deficiencies. In addition, ALP knockout mice models have shown that mice are born with normal bone but acquire defects in bone mineralization shortly thereafter (Hui and Tenenbaum, 1998; Narisawa et al., 1997). Therefore, the activity and localization of ALP have been considered a valuable indicator for bone development and differentiation (Miao and Scutt, 2002).

ALP enzyme activation requires homodimerization at the membrane (Hoylaerts et al., 1997). One mechanism by which a cell can concentrate or sequester proteins is by utilizing lipid rafts. Our data illustrate that ALP is localized to and active at osteoblastic lipid rafts and disruption of the rafts reduces both ALP activity and mineralization. Lipid rafts function to organize the plasma membrane into a series of discrete microdomains (Galbiati et al., 2001). These platforms can then conduct a number of cellular functions. One role described for lipid rafts at the cell surface is to organize proteins involved in signal transduction pathways (Brown and London, 1998). For example lipid rafts have been shown to mediate IgE signaling that occurs during the allergenic immune response (Sheets et al., 1999; Simons and Toomre, 2000). In this process, rafts cluster following activation and receptor crosslinking, which results in redistribution of raft components leading to transmembrane signaling. Subsequent studies determined that IgE signaling was abolished with the sequestration of cholesterol (Stauffer and Meyer, 1997). Raft microdomains are also utilized during signaling from Ras (Roy et al., 1999), Hedgehog (Rietveld et al., 1999) and T-cell antigen receptors (Janes et al., 2000). Our studies provide evidence that ALP is localized to osteoblastic lipid rafts, where we suggest its dimerization state and thus activity may be regulated. In addition, many signaling molecules involved in bone differentiation such as src, hedgehog and protein kinase C have been localized to lipid rafts in other cell types. The effects of these and other signaling molecules have been well characterized, however the complete signaling cascades responsible for bone mineralization remain incompletely understood (Eriebacher et al., 1995; Ferguson et al., 1998; Marzia et al., 2000; Simons and Toomre, 2000). We are currently evaluating the role of lipid rafts in signal transduction during differentiation-induced mineralization.

In addition to mediators of signal transduction, rafts have been implicated in protein sorting particularly through the secretory and endocytic pathways (Brown and London, 1998). Cholesterol- and sphingolipid-containing rafts initially assemble in the Golgi where protein association is important for polarized delivery of these proteins to the cell surface. Small vesicles containing various proteins and lipids are released from the Golgi and travel predominantly toward the plasma membrane where they deliver specific cargo (Simons and Toomre, 2000). In epithelial cells, rafts are found to cluster at the apical plasma membrane, whereas in fibroblasts, rafts are distributed throughout the cell surface (Simons and van Meer, 1988). Therefore, lipid rafts may be an appropriate physical entity for the temporal and spatial regulation of proteins required to initiate mineralization.

Anx2 is one possible molecular component involved in regulating membrane events. Several lines of research have implicated anx2 in the organization and dynamics of lipid rafts through its ability to bind membranes and the cytoskeleton. In smooth muscle and mammary epithelial cells, anx2 has been described as one of a few proteins found to link the membrane to the cytoskeleton at raft microdomains (Babiychuk and Draeger, 2000; Oliferenko et al., 1999). Additional studies illustrated that anx2 was clustered to lipid rafts at sites of bacterial adherence, possibly serving to stabilize the rafts

through actin binding (Zobiack et al., 2002). During osteoblastic mineralization, we propose that lipid rafts coalesce in order to activate enzymes (such as ALP) or signaling cascades (such as src). Anx2 may function to stabilize the microdomains and link them to the cortical actin cytoskeleton. Alternatively, anx2 may facilitate the sequestration of small membrane rafts into larger functional microdomains.

An interesting observation is the similarity between the lipid and protein profiles of lipid rafts and chondrocytic MV. Both structures are known to be enriched in cholesterol, shingolipids and phosphatidylserine, in addition to proteins such as annexins and ALP (Anderson, 1995; Babiychuk and Draeger, 2000; Brown and London, 1998; Genge et al., 1990; Pierini and Maxfield, 2001; Saslowsky et al., 2002; Simons and Toomre, 2000; Wuthier, 1976). The mechanism by which the necessary proteins are clustered together at the sites of MV is not well understood; and while it has been established that MV are released from 'specialized domains' at the plasma membrane, the mechanisms responsible for the production and release of MV remain unclear (Leach et al., 1995). We hypothesize that lipid rafts may represent the membrane microdomains to which mineralization-specific proteins are recruited prior to the release of MV. Therefore, anx2 stabilization of rafts and sequestration of appropriate proteins through phospholipids and actin binding may facilitate mineralization in both chondroblasts and osteoblasts.

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