Annexin II is a heterotetramer, consisting of two p11 and two p36 kDa subunits, that is produced by osteoclasts and stimulates osteoclast formation. However, its receptor is unknown. We showed that annexin II binds to normal primary human marrow stromal cells and the Paget's marrow-derived PSV10 stromal cell line to induce osteoclast formation. 125I-labeled annexin II binding assays with PSV10 cells demonstrated that there was a single class of annexin II receptors with a K_d of 5.79 nM and B_max of 2.13 x 10^5 receptors/cell. Annexin III or annexin V did not bind this receptor. Using 125I-labeled annexin II binding to screen NIH 3T3 transfected with a human marrow cDNA expression library, we identified a putative annexin II receptor clone, which encoded a novel 26 kDa type I membrane receptor protein when expressed in HEK 293 cells. HEK 293 cells transformed with the cloned annexin II receptor cDNA showed a similar binding affinity to annexin II as that observed in PSV10 cells. Chemical cross-linking experiments with biotinylated annexin II and intact PSV10 cells identified a 55 kDa band on Western blot analysis that reacted with both an anti-p11 antibody and streptavidin, but not anti-p36 antibody. A rabbit polyclonal antibody raised against the putative recombinant annexin II receptor also recognized the same 26-kDa protein band detected in PSV10 cells. Importantly, the annexin II receptor antibody dose-dependently blocked the stimulatory effects of annexin II on human osteoclast formation, demonstrating that the receptor mediates the effects of annexin II on osteoclast formation.

The osteoclast (OCL) is the primary cell responsible for bone resorption. The molecular and cellular events involved in osteoclastogenesis are a topic of intensive investigation (1). Factors that regulate OCL precursor differentiation can be produced systematically, such as 1α,25-(OH)2D3, calcitonin, and parathyroid hormone, or be produced locally, such as interleukin 6 (IL-6) and receptor activator of nuclear transcription factor κB ligand (RANKL), in the bone microenvironment by osteoblasts, stromal cells and immune cells (2). In addition, autocrine factors that positively or negatively regulate osteoclastogenesis and OCL activity have been identified. These include annexin II (3), IL-6 (4), OCL stimulatory factor (OSF) (5), eosinophil chemotactic factor-L (ECFL) (6), and ADAM8 (7), which stimulate OCL formation, and OCL inhibitory peptide-1 (OIP-1) and -2 (OIP-2) (8), which inhibit OCL formation. Except for IL-6, the receptors for these autocrine factors produced by OCL have not been clearly identified.

Annexin II (AX-II) is a member of a family of twenty proteins that bind to anionic phospholipid surfaces in the presence of calcium. Each annexin has a unique N-terminal amino acid sequence with a conserved C-terminus (9). The structures of annexins have been well defined, but their precise functions are still being elucidated (10). Annexin II has been implicated as an...
intracellular protein in endocytosis and exocytosis (11, 12). As an extracellular protein, AX-II participates in cell-cell adhesion, and acts as a potential receptor for tissue plasminogen activator (13), tenasin-C and human cytomegalovirus (14). Although its role as a receptor for 1α,25-(OH)2D3 is controversial (15), AX-II heterotetramer has been identified as a receptor for the plasmin-induced signaling in human peripheral monocytes (16). AX-II can exist as a monomer, homodimer, or heterotetramer comprised of two p36 molecules and two p11 molecules [(p36)2(p11)2]. The heterotetramer is the predominant species in all tissues and cells and appears to be the active form of AX-II (17). The p11 protein (S100A10), also known as calpactin-I light chain, is a member of S-100 EF hand superfamily of Ca2+-binding proteins. However, p11 has crucial amino acid deletions and substitutions that render its Ca2+-binding sites inactive (18). It serves as the regulatory unit of the annexin II heterotetramer (19). Differential expression of p11 and the annexin II subunit (p36) may be associated with different cellular functions (20).

We identified the annexin II heterotetramer as an autocrine/paracrine factor that stimulates OCL formation in both human and murine marrow cultures (21). Further, we demonstrated that the stimulatory effects of AX-II on osteoclastogenesis were mediated indirectly by inducing production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and RANKL by marrow stromal cells and CD4+ T-cells (3, 22). Although AX-II can bind to phospholipid surfaces nonspecifically, we found that fluoresceinated AX-II preferentially bound to marrow stromal cells and CD4+ T-cells, but not to CD8+ T-cells, and that this binding was Ca2+-dependent and could be completely competed by unlabeled AX-II. These results suggested that CD4+ T-cells and marrow stromal cells express a putative AX-II receptor. Further supports for an AX-II receptor include that AX-II binds to endothelial cells in a saturable equilibrium-based and calcium-dependent manner, and EGTA can strip AX-II from the cell surface (23). In addition, a potential intracellular AX-II membrane receptor has been reported but not characterized (24).

In this study, we demonstrate that the AX-II tetramer (AXIIIt) binds to a single class of high affinity receptors on the surface of both primary normal human marrow stromal cells and the PSV10 marrow stromal cell line derived from a Paget’s disease patient. Using expression library cloning with a human bone marrow library, we identified a cDNA clone that encoded the annexin II receptor that bound to AX-II through the p11 subunit. HEK 293 cells transformed with this cDNA clone bound to AX-II with a high affinity similar to that observed in PSV10 cells. Further, a polyclonal antibody raised against the putative receptor blocked AX-II induced p44/42 phosphorylation and OCL formation in vitro.

Experimental Procedures

Materials – The 23c6 monoclonal antibody (mAb) that identifies vitronectin receptors on OCLs was provided by Dr. Michael Horton (St. Bartholomew’s Hospital, London, United Kingdom). Highly purified bovine lung annexin II tetramer (AXIIIt) was purchased from BIODESIGN International (Saco, ME). Goat anti-human osteoprotegerin (OPG) neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). Anti-p36 and anti-p11 mAbs were purchased from BD Transduction Laboratories (Lexington, KY). Dulbecco’s Modified Eagle Medium (DMEM) and Alpha Minimum Essential Medium (α-MEM) were purchased from Invitrogen (Carlsbad, CA). Fetal calf serum (FCS) and horse serum were purchased from JRH Sciences (Lenexa, KS).

Human bone marrow cultures and cell lines – After obtaining informed consent, 2-ml of bone marrow were aspirated from the posterior-superior iliac crest of healthy normal volunteers into a 20-ml syringe containing 1-ml of α-MEM and 100 units of preservative-free heparin. This protocol was approved by the Institutional Review Board of the University of Pittsburgh. The bone marrow was then processed as described (25). Briefly, the mononuclear cell fraction was obtained by density gradient centrifugation using Ficoll-Hypaque (Sigma, St. Louis, MO) and the mononuclear cells were incubated at 37°C in 20% FCS α-MEM in 100-mm tissue culture dishes (5 x 10⁶ cells/ml) overnight. The nonadherent cells were used in cultures as the source of immature precursors for OCL formation. The adherent cells were kept in 10% FCS α-MEM and used as the source of human primary bone marrow stromal cells.
(hBMSC) (26). These cells did not contain detectable hematopoietic cells. SAKA-T and PSV10 are human stromal cell lines derived from normal bone marrow and marrow from a patient with Paget's disease respectively.

**Immunocytochemistry** – Normal primary human stromal cells were seeded in 4-well LabTek (Nunc, Inc., Naperville, IL) chamber slides overnight. These cells were incubated in the presence of 2 µg/ml of biotinylated AX-II with or without a fivefold excessive unlabeled AX-II, then incubated with streptavidin-conjugated peroxidase, and finally exposed to DAB substrate.

**Iodination of the annexin II tetramer (AXII) and Equilibrium binding assays** – Soluble AXII was iodinated using the 125I-Bolton-Hunter Reagent (NEN Life Science Products, Boston, MA) according to the manufacturer’s instructions. In some experiments, the 125I-labeled AXII was purchased from Perkin Elmer (Wellesley, MA). The iodinated AXII (125I-AXII) had a specific activity of 172.3 Ci/mmol. The equilibrium binding assays were performed as follows. 5.4 x 10^4 PSV10 cells/well were seeded in 24-well plates (Corning Incorporated, Corning, NY) overnight. On the next day, the cells were incubated with increasing amounts of 125I-AXII in the presence or absence of 100-fold excessive unlabeled AXII (cold AXII) in annexin binding buffer (BD Clontech, Palo Alto, CA) on ice for 3 hours. Cells were washed extensively with PBS containing 1 mM CaCl₂, detached from the dish by scraping, and collected by centrifugation. Pellets were resuspended in 470 µl of 40 mM HEPES (pH 7.4), 5 µl of 100x protease inhibitor cocktail (Calbiochem, San Diego, CA), 25 µl of 100 mM Dithio-bis-maleimidoethane (DTME) (Pierce, Rockford, IL), and incubated for 2 hours at room temperature. The cells were collected by centrifugation, resuspended in 396 µl of buffer A (20 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA) and 1 µl of 100x protease inhibitor cocktail for 20 minutes on ice. After centrifugation, the reaction mix was resuspended in 99 µl of buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) and 1 µl of 100x protease inhibitor cocktail for 20 minutes on ice, and then centrifuged and resuspended in 50 µl of lysis buffer (1% TritonX-100, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 1 µl of 100x protease inhibitor cocktail. Ten micrograms of protein from each sample were suspended in a native electrophoresis loading buffer (BioRad, Hercules, CA). The protein bands were resolved on 12% polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schnell, Keene, NH) electronically. Nonspecific binding was blocked with 5% nonfat milk/TBS-T. The membranes were exposed to horse-radish peroxidase (HRP)-conjugated streptavidin directly, or exposed sequentially to anti-p36 or anti-p11 receptor cDNA or pcDNA3.1 empty vector were generated using standard molecular techniques. Then 1.0 x 10^5 of the stably transfected HEK 293 cells were seeded in 24-well tissue culture plates overnight. On the next day, the equilibrium binding assays were performed as outlined above.

**Biotinylation of annexin II tetramer (Bio-AXII) and cross-linking of Bio-AXII to PSV10 cells** – Biotylated AXII was prepared using the BiotinTag Micro-biotinylation kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. The biotinylation was confirmed by Western blot analysis. PSV10 cells (2.5 x 10^6 cells/dish) were seeded in 100-mm tissue culture dishes overnight and then incubated with or without 2 µg of Bio-AXII suspended in 4 ml of annexin binding buffer in the presence or absence of 15 µg of unlabeled AXII for 3 hours on ice. Cells were washed 3 times with PBS containing 1 mM CaCl₂, detached from the dish by scraping, and collected by centrifugation. Pellets were resuspended in 470 µl of 40 mM HEPES (pH 7.4), 5 µl of 100x protease inhibitor cocktail (Calbiochem, San Diego, CA), 25 µl of 100 mM Dithio-bis-maleimidoethane (DTME) (Pierce, Rockford, IL), and incubated for 2 hours at room temperature. The cells were collected by centrifugation, resuspended in 396 µl of buffer A (20 mM HEPES-KOH pH 7.9, 1.5 mM MgCl₂, 0.2 mM EDTA) and 1 µl of 100x protease inhibitor cocktail for 20 minutes on ice. After centrifugation, the reaction mix was resuspended in 99 µl of buffer C (20 mM HEPES-KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) and 1 µl of 100x protease inhibitor cocktail for 20 minutes on ice, and then centrifuged and resuspended in 50 µl of lysis buffer (1% TritonX-100, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and 1 µl of 100x protease inhibitor cocktail. Ten micrograms of protein from each sample were suspended in a native electrophoresis loading buffer (BioRad, Hercules, CA). The protein bands were resolved on 12% polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schnell, Keene, NH) electronically. Nonspecific binding was blocked with 5% nonfat milk/TBS-T. The membranes were exposed to horse-radish peroxidase (HRP)-conjugated streptavidin directly, or exposed sequentially to anti-p36 or anti-p11 receptor cDNA or pcDNA3.1 empty vector were generated using standard molecular techniques. Then 1.0 x 10^5 of the stably transfected HEK 293 cells were seeded in 24-well tissue culture plates overnight. On the next day, the equilibrium binding assays were performed as outlined above.
mAb and then HRP-conjugated goat anti-mouse IgG (Bio-Rad). Positive bands were visualized with the enhanced chemiluminescence (ECL) (Amer sham). The nitrocellulose membrane was washed with Tris-buffered saline with 0.1% Tween three times between incubation steps.

**Annexin II Receptor cloning by expression library screening and gene mapping** – A human bone marrow cDNA expression library (Invitrogen) was divided into 20 pools each containing approximately 2000 clones. Purified plasmid DNA (10 µg) obtained from each pool was transiently transfected into NIH3T3 cells plated in 24-well plates (2 x 10⁴ cells/well) in DMEM supplemented with 10% FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin using LipofectAMINE (Invitrogen). After 48 h, the cells were incubated with ¹²⁵I-AXIIt on ice for three hours. The cells were washed 5x with 1mM CaCl₂/ PBS containing 2% horse serum, and then lysed in 10 N NaOH. The cell lysates were collected, and ¹²⁵I-AXIIt binding activity was measured with a γ-counter. The plasmid pool showing the highest binding activity was selected. This positive plasmid pool was sub-fractionated until a single putative positive cDNA clone (88-5-D1), which significantly increased ¹²⁵I-AXIIt binding when transfected into NIH3T3 cells, was identified. The specificity of ¹²⁵I-AXIIt binding to NIH3T3 cells transfected with the candidate clone was further confirmed by inhibition of ¹²⁵I-AXIIt binding by addition of 100x cold AXIIt, but not by 100x cold annexin V. Nucleotide sequence of the putative AX-II receptor cDNA (1182 bp) was determined (GenBank Accession No. NM_001014279).

Chromosomal assignment of human annexin II receptor was undertaken by polymerase chain reaction analysis of a somatic cell hybrid panel (Invitrogen). The primers were CCGAGTCTACTGGCAAAACG (sense) and GCCTTCTGCTGCTATCTAAG (antisense). The PCR amplicon was 356 bp.

**Mammalian Expression plasmids** – To construct an N-terminus Flag-tagged vector, the annexin II receptor coding sequence was amplified with primers

\[
\begin{align*}
\text{GATCCTCGAGATGGACTACAAGGACGACG} & \quad \text{(forward)} \\
\text{GTACTCTAGACTAAGGCTGTTAGCTCCAC} & \quad \text{AGAT (reverse)}
\end{align*}
\]

using Pfx DNA polymerase (Invitrogen). The underlined sequence in the forward primer encoded the flag tag (DYKDDDDK). The PCR amplicon was cloned into pcDNA3.1(+) (Invitrogen) in frame between the XhoI and XbaI restriction sites. The constructed vector was confirmed by DNA sequencing.

**Annexin II receptor antibody generation** – An annexin II receptor specific antibody was raised in rabbits against the C-terminus 70-193 amino acids of the recombinant AX-II receptor protein expressed in 293 cells (Alpha Diagnostic International, San Antonio, TX). Rabbit IgG from anti-sera showing immunoreactivity to the recombinant annexin II receptor was purified with the HiTrap Protein A HP kit (Amer sham Pharmacia, Piscataway, NJ) and concentrated for immunoblotting and bioassays. The antibody preparation did not contain any preservatives.

**OCL formation assays** – OCL formation assays were performed as described previously (28-31). Briefly, 1 x 10⁵ nonadherent human bone marrow mononuclear cells were seeded in 0.2 ml of 20% horse serum and 10 ng/ml recombinant M-CSF in α-Minimal Essential Medium (α-MEM) with the indicated factors in 96-well flat-bottom tissue culture plates, and incubated in a humidified 37°C tissue culture incubator in an atmosphere of 5% CO₂. The cultures were replenished a half volume of the media containing the indicated factors twice per week. After three weeks of incubation, the cells were fixed with 2% formaldehyde (Sigma, St. Louis, MO) and stained with 23c6 mAb that identifies vitronectin receptors on OCL (27). Positive reactions were visualized with the VECTASTAIN ABC-AP kit and VECTOR red alkaline phosphatase substrate (Vector Laboratories). Cells that contained three or more nuclei and reacted to 23c6 mAb were scored as OCL-like multinucleated cells.

**Effects of annexin II receptor antibody on marrow stromal cells** – Normal primary human bone marrow stromal cells were plated in 6-well plates overnight (1 x 10⁶ cells/well). The cells were treated with AXIIt 250 ng/ml in the presence or absence of the annexin II receptor (AXIIR) polyclonal antibody (0 to 5 µg/ml) for 24 hours. RT-PCR was used to examine RANKL and OPG in RNA expression. Total RNA was purified with RNA-Bee (Tel-Test, Inc., Friendswood, TX). Two µg total RNA was reverse transcribed in 20 µl reaction volume, and two µl of the RT reaction
mix was used for each PCR reaction. Both RNA reverse transcriptase and Taq DNA polymerase were obtained from Invitrogen. The PCR primers were for RANKL, ATAGAATATCAGAAGATGGCACTC (forward) and TAAGGAGGGTGGAGACCTCG (reverse), and for OPG, GTGTCTTTGGTCTCCTGCTAA (forward) and GGGCTTTGTTTTGATGTTTC (reverse), for GAPDH ACCACAGTCCATGCCATCAC (forward) and TCCACACCTGTGGCTGTA (reverse). The parameters for amplification were 94°C for 30 seconds, 60°C for 1 minute for 30 cycles (except 25 cycles for GAPDH) and 60°C for 7 minutes as the final elongation step for all reactions.

Effects of the annexin II receptor antibody on OCL formation – Human primary bone marrow mononuclear cells from healthy donors were used for OCL formation assays as described above. The cells were treated with AXIIIt (250 ng/ml) with or without AXIIIR polyclonal antibody (0 to 5 µg/ml). At the end of the 3-week incubation, OCL-like cells were scored. Treatment with RANKL (20 ng/ml) and rabbit IgG (1 µg/ml) were used as the positive and negative control respectively.

Tissue distribution of the annexin II receptor – Human tissue cDNA panels were purchased from Clonetech (Palo Alto, CA). RT-PCR was used to determine the relative abundance of AXIIR transcripts among tissues. The primers were TTGGGATTCCGCAGAGGTGG (forward) and CAGCACGCCCAGAGTACAGAGAA (reverse). The parameters were 94°C 15 seconds and 68°C for 90 seconds for 35 cycles and 60°C 7 minutes for the final elongation.

Statistic analysis – Results were expressed as the mean ± SEM. Prism (GraphPad Software Inc, San Diego, CA) was used for statistic analysis, nonlinear curve fitting. Differences between treatment groups were tested by student t-test (for two groups) or two-way ANOVA (three or more groups). Differences were considered significant for p < 0.05.

RESULTS

Human marrow stromal cells express a putative annexin II receptor: We previously demonstrated that AX-II binds to CD4+, but not CD8+ T-cells (3) and to normal primary marrow stromal cells and the marrow stromal cell line PSV10 cells, and this binding could be completely competed with excessive annexin II. These data suggested that annexin II may utilize a receptor to mediate its effects on OCL formation. Therefore, we determined if marrow stromal cells expressed an AX-II receptor. As shown in Fig. 1A, biotinylated AXIIt bound to normal human marrow stromal cells (Fig 1A) and this binding was abolished by the addition of excessive unlabeled AXIIIt. To determine the binding specificity of AXIIt to its putative receptor, 125I-labeled AXIIt (125I-AXIIIt) was incubated with PSV10 cells or human primary bone marrow stromal cells with 80-fold excessive unlabeled annexin II, annexin IV or annexin V respectively. Binding of 125I-AXIIIt to PSV10 cells (Fig. 1B) or normal primary bone marrow stromal cells (Fig. 1C) was significantly decreased by co-incubating with the unlabeled AXIIIt. In contrast, excessive unlabeled annexin III or annexin V did not compete for the 125I-AXIIIt binding. Therefore, binding of AX-II to the surface of PSV10 cells and primary stromal marrow cells was saturable and specific.

We then performed 125I-AXIIIt equilibrium binding assays using PSV10 cells to determine the number of receptors/cell and the binding affinity of AXIIIt for its putative receptor. When PSV10 cells were treated with increasing amount of 125I-AXIIIt, saturable specific binding of AXIIIt to PSV10 cells surface was observed (Fig. 1D). As shown on the Scatchard plot (Fig. 1E), a single class of high affinity receptors was detected on PSV10 cells, with a Kd of 5.79 nM and Bmax of 2.13 x 10⁵/cell.

Identification of the PSV10 cells membrane protein that is cross-linked to biotinylated AXIIIt (bio-AXIIIt): Biotinylation of AXIIIt resulted in labeling of both the p36 and p11 subunits. Western blot analysis showed that p36 had a much higher molar ratio of biotinylation than that of p11 (data not shown). To further confirm that an annexin II receptor was expressed on the surface of PSV 10 cell, chemical cross-linking experiments were performed with biotinylated AXIIIt. Intact PSV 10 cells were incubated with medium alone, biotinylated AXIIIt, or biotinylated AXIIIt plus excessive unlabeled AXIIIt, then cross-linked, lysed, and subjected to Western blot analysis. Blotting with streptavidin revealed two specific bands, a 55- and a 36-kDa. The 36-kDa band was the biotinylated p36 subunit and the 55-kDa cross-
linked product contained the putative receptor. The biotinylated 55-kDa band was not detected when excessive unlabeled AXIIt was added prior to the cross-linking reactions (Fig. 2A).

To further identify the composition of the 55-kDa band, we blotted the membrane with mouse anti-p36 or anti-p11 mAb respectively. As shown in the left panel of Fig 2B, the anti-p36 antibody only recognized the 36-kDa band but not the 55-kDa band (Fig. 2B right panel). In contrast, the 55-kDa band reacted only with the anti-p11 antibody. Thus, the p11, not p36, subunit of the AXIIt was the primary portion of the AXIIt that bound to the receptor on PSV10 cells.

Expression library cloning of AXII receptor and gene mapping: To confirm that the protein we detected on PSV10 cells was an AX-II receptor, we used an expression library cloning approach to identify the mRNA that encoded the annexin II receptor. The identified cDNA sequence (1182 bp) was submitted to the GenBank® with accession No. NM_001014279. As shown in Fig 2C, Western blot analysis of lysates from SAKA-T cells transfected with the N-terminal Flag-tagged AXIIIR clone and then cross-linked to AXIIt showed a similar 55 kDa band that reacted to both streptavidin and anti-Flag antibody. To further determine if a similar receptor was expressed in other stromal-derived cells, Northern blot analysis was performed with the labeled AXIIIR cDNA. Northern blot analysis revealed the predicted 1.2 kb band among the human bone marrow cells, human osteoblastic-like cells (MG63), and stromal cell lines derived from normal (SAKA) and Paget’s patient (PSV10) respectively (Fig 2D). Further, the cloned cDNA or pcDNA3.1 empty vector were transfected into HEK 293 cells and stable expressing cells were generated. We then performed radioligand binding assays with the HEK 293 transformants. As shown in Fig 2E, 125I-AXIIt bound to the annexin II receptor cDNA expressing HEK 293 cells with a high affinity (Kd = 6.3 nM). Taken together, the cloned cDNA sequences encode a protein that specifically bound to AX-II tetramer with a similar affinity as the native receptor.

To identify the AXIIIR gene chromosomal location, PCR analysis of a mouse/human somatic cell hybrid panel was performed. As shown in Fig 3A, the specific PCR amplicon using the primer set was detected only from the hybrid containing human chromosome 5. Further, bone marrow mRNAs from hamster, mouse, and rat were used as controls. The specific PCR amplicon was only detected in the human sample (Fig 3B).

Tissue distribution of the annexin II receptor: The tissue distribution of the annexin II receptor was analyzed with a commercial cDNA panel. As shown in Fig 3C, most tissues/organs expressed the annexin II receptor. Among them, lymphocytes had the most abundant expression. Surprisingly both resting CD4+ and CD8+ T-cells expressed the AXIIIR, but the expression of the receptor markedly decreased in activated CD8+ T-cells.

Effects of annexin II receptor (AXIIIR) antibody on bone marrow stromal cells and OCL formation: To determine whether the AXIIIR we identified mediated the biologic effects of AX-II, we tested the effects of blocking the annexin II receptor on the OCL formation by marrow cells treated with AXIIt plus increasing amounts of the annexin II receptor antibody. The AX-II receptor antibody blocked the stimulatory effect of AXIIt on the OCL formation dose-dependently (Fig 4A). Importantly, low concentrations of the annexin II receptor polyclonal antibody significantly inhibited OCL formation induced by AXIIt. Rabbit IgG did not have any inhibitory effects on OCL formation. As a control, the AXIIIR antibody preparation itself did not show toxic effects in OCL culture since it did not inhibit OCL formation induced by RANKL. These data suggested that the stimulatory effects of AXIIt on OCL formation were mediated by the annexin II receptor.

We previously reported that AX-II stimulate OCL formation by activating the MAP kinase pathway. Therefore, we examined the effects of annexin II receptor antibody in the presence of AX-II on stromal cells. As expected, the annexin II receptor antibody blocked the p44.42 phosphorylation stimulated by AX-II dose-dependently (Fig. 4B). We then determined if the inhibitory effects of the antibody were due to blocking AX-II or inducing an inhibitor of OCL formation such as OPG. RT-PCR analysis of primary human bone marrow stromal cells treated with the AXIIIR polyclonal antibody revealed that OPG mRNA expression was increased dose-dependently at the protein level as well (Fig. 4D). Further, when primary marrow stromal cells were incubated with the AXIIIR antibody, OPG expression levels increased dose-dependently (Fig
Since the AXIIR antibody increased OPG mRNA expression in human stromal cells, we then examined if an OPG neutralizing antibody could reverse the effects of AXIIR antibody on AX-II induced osteoclastogenesis. The OPG neutralizing antibody only partially reversed the inhibitory effects of the AXIIR antibody on OCL formation approximately 30% (Fig 4E).

DISCUSSION

We previously demonstrated that annexin II selectively bound to CD4+ cells and marrow stromal cells. This binding was saturable and could be competed with excess AXIIIt. The data suggest the possibility of a surface annexin II receptor. Our current studies demonstrate that there is a specific surface AXIIIt receptor on marrow stromal cells. We demonstrated that biotinylated AXIIIt bound to normal human stromal cells (Fig. 1A) and this binding could be competed off with excessive unlabeled AX-II. Competitive binding assays using 125I-labeled AXIIIt on the human PSV10 stromal cell line (Fig. 1B) and primary marrow stromal cells (Fig. 1C) demonstrated the excess AXIIIt, but not annexin III and V, blocked 125I-AXIIIt binding to the cells. Further, equilibrium bind assays indicated that the binding of AX-IIIt was to a single class of high affinity receptors (Fig. 1D and 1E). Moreover, specific binding of AXIIIt was demonstrated with the chemical crosslinking. DTME (Dithio-bis-maleimidoethane) is a sulfhydryl-reaction cross-linker. After the crosslinking and Western blot, a protein complex was identified with an approximate size of 55-kDa. Further analysis showed that AXIIIr binds the p11 subunit of AXIIIt but not p36 subunit of AXIIIt. The calculated size for the cloned annexin II receptor is around 26 kDa, and the crosslinked protein complex is approximately 55-kDa. Although p11 exists as a dimer and connects one p36 on each side symmetrically (32), it is not clear whether the 55-kDa band contains one or two molecules of p11. Our results suggest that the cross-linked protein complex contains two molecules of p11. Further, HEK 293 cells stably expressing the annexin II receptor cDNA exhibited a high binding affinity for AXIIIt, similar to that observed with the Paget’s disease derived stromal cell line (PSV10). It is well documented that plasmin interact with AX-II tetramer by activating a series of proteolytic changes in the annexin II subunits, which initiate downstream signaling and final biological responses (16). Therefore, it is possible that these proteolytic fragments of AX-II tetramer could act as a receptor for AXII. However, alignment of the cloned annexin II receptor cDNA sequence with both p36 and p11 cDNA sequences did not show any significant similarities. Taken together, we have identified a unique annexin II surface receptor in human stromal cells.

Annexin II receptor mRNA transcripts were detected in most tissues examined (Fig. 3C), except the heart, the brain, and skeletal muscle. Interestingly, activated CD8+ T-cells have decreased expression of annexin II receptor compared to resting CD8+ T cells. These data are in agreement with our previous finding that annexin II preferably bound to CD4+, but not CD8+ T-cells. The significance of the tissue distribution of the annexin II receptor transcripts remains to be determined.

Our functional studies demonstrate that the annexin II receptor mediates the effects of AX-II on OCL formation (Fig. 5A). We reported that AX-II stimulates OCL formation via the MAP kinase pathway by phosphorylating p44/42. To further confirm the cloned AX-II receptor, we used the polyclonal annexin II receptor antibody to block the stimulatory effect of AX-II on human primary stromal cells. p44/42 phosphorylation was blocked dose-dependently by the AXIIR antibody, consistent with our previous report.

The RANK/RANKL/OPG pathway is a pivotal regulator of bone metabolism. OPG is a secreted decoy receptor that binds RANKL (33). In human bone marrow stromal cell cultures, treatment with the annexin II receptor antibody increased OPG expression dose-dependently (Fig 4C & 4D). Importantly, these data, again, confirmed the existence of the annexin II receptor, since the polyclonal antibody was raised specifically against the C-terminal 70-193 amino acids of the annexin II receptor. We then examined the effects of annexin II receptor antibody on bone marrow cultures. As shown in Fig. 4A, annexin II receptor polyclonal antibody significantly inhibited OCL formation induced by AX-II at a concentration as low as 100 ng/ml. Although, the AXIIR antibody induced expression of OPG, an excess of anti-OPG only partially reversed the
inhibitory effects of the AXIIR antibody. These data demonstrated that the stimulatory effects of annexin II in the bone marrow culture were mediated by the annexin II receptor. Taken together, these data demonstrate that this protein is a specific surface receptor for annexin II.

Structural analysis with SOSUI (34, 35) and TMPred (36) predicted that the AXIIR is a type I transmembrane protein with one transmembrane domain. Interestingly, only four amino acids are present in the cytoplasmic tail. These results suggest that the AXIIR receptor need a binding partner(s) and/or docking proteins for proper signaling, as does the fibroblast growth factor (FGF) receptor which signals through Frs2 (Fibroblast growth factor receptor substrate 2) (37). We previously reported that annexin II upregulates RANKL via the MAPK pathway (22), suggesting that the binding of the AXIIR to its binding partner activates MAP kinase.

In conclusion, we have identified the first surface receptor for an annexin and showed that it mediates the stimulatory effects of AX-II on osteoclastogenesis. Identification of the AX-II receptor should permit further characterization of the signaling pathways utilized by AX-II, identify its binding partners and allow us to screen phage display libraries for small molecules that induce or block signaling by this receptor. Identification of these small molecules will permit testing their potential utility in diseases associated with abnormalities in OCL activity such as osteoporosis and bone metastasis.

REFERENCES

FOOTNOTES

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1The abbreviations used are: AX-II, annexin II; AXIIIt, annexin II-p11 heterotetramer; GM-CSF, granulocyte-macrophage colony-stimulating factor, MNC, multinucleated cell; OPG, osteoprotegerin; OCL, Osteoclast; PBS, phosphate buffered saline; RANKL, receptor activator of nuclear transcription factor κB ligand, TBST, Tris-buffered saline with 0.1% Tween.

FIGURE LEGENDS

Fig. 1. Annexin II receptor expression on normal human bone marrow stromal cells and PSV10 cells. A, Normal primary human marrow stromal cells were incubated in the presence of 2 µg/ml of Bio-AXIIIt with or without an eighty-fold excess of unlabeled AXIIIt, then incubated with horseradish peroxidase-conjugated streptavidin, and finally exposed to DAB substrate. Dark brown staining denotes a positive reaction. PSV10 cells (B) or normal primary human marrow stromal cells (C) were incubated in the presence of 125I-AXIIIt with or without an 80-fold excess of unlabeled annexin II tetramer, annexin III, or annexin V. After extensive washing, the radioactivity in the samples was detected with a γ-counter. Each assay was performed in triplicate. Similar results were seen in three independent experiments. Results from one typical experiment are shown and represent as the mean ± SEM. * p < 0.01. D, Binding of 125I-
AXIIIt to PSV10 cells was assayed as described in Experimental Procedures. Total binding represents bound $^{125}$I-AXIIIt in the absence of unlabeled AXIIIt, and non-specific binding represents bound $^{125}$I-AXIIIt in the presence of 100-fold excess of unlabeled AXIIIt. Specific binding was calculated by subtracting the non-specific binding from the total binding. All assays were performed in duplicate. Similar results were seen in three independent experiments. E, Scatchard plot for the binding assay for the panel D.

Fig. 2 Identification of the PSV10 cell membrane protein cross-linked to AXIIIt and confirmation of the cloned putative AX-II receptor cDNA. Biotinylated AXIIIt was incubated with intact PSV10 with or without a 100-fold of excess of unlabeled AXIIIt, cross-linked with DTME. The cells were washed extensively and the lysates were subjected to Western blotting analysis. A, Horseradish peroxidase conjugated streptavidin blotting revealed a band of 55 kDa complex, the non-crosslinked biotinylated p36 subunit was also detected. B, To further identify the proteins in the 55 kDa complex, monoclonal antibodies against p36 or p11 subunits were used to blot the membrane. Positive bands were visualized with the ECL. C, the N-terminal Flag-tagged AXIIIR vector was transiently transfected into SAKA-T cells. After 2-day incubation, the cells were incubated with Bio-AXIIIt and cross-linked with DTME. The cell lysates were then subjected to Western blot analysis. D, Northern blot analysis of the AXIIIR mRNA in marrow stromal-derived cells. E, $^{125}$I-labeled AX-II equilibrium binding assay with HEK 293 cells stably expressing the AX-II receptor cDNA or empty vector. The specific binding was calculated by subtracting binding of AX-II to empty vector expressing cells from that of AX-II receptor expressing cells. The Scatchard plot is shown as an insert. Similar results were observed in three independent experiments. Results from one experiment are shown.

Fig. 3 Chromosomal location of the annexin II receptor gene and tissue distribution of the annexin II receptor mRNA. A, PCR analysis of a human somatic cell hybrid panel showed the specific amplicon was only detected in the hybrid that contained human chromosome 5. B, Bone marrow mRNAs from hamster, mouse, and rat were used controls. The specific PCR amplicon was only detected in human sample. C, The experiment was performed twice, using commercial tissue cDNA panels. Similar results were seen in two independent experiments. GAPDH was used as the internal loading control. According to the vendor, the GAPDH expression level could vary among tissues despite normalization.

Fig. 4 Effects of AXIIIR antibody on human stromal cells and on OCL formation. A, normal nonadherent bone marrow mononuclear cells (1 x 10$^6$ cells/well) were plated in 96-well tissue culture plate. The cells were treated with AXIIIt (250 ng/ml) with or without AXIIIR antibody (10 ng/ml to 1,000 ng/ml) to induce OCL formation as described. RANKL (20 ng/ml) and rabbit IgG were used as positive and negative controls respectively. Similar results were seen in four independent experiments. Results from one typical experiment are shown and are presented as the mean ± SEM. * p < 0.05 compared to controls. B, human primary stromal cells were serum starved for 1 hour and incubated with the AXII receptor antibody in the presence of 250 ng/ml of AX-II for 15 minutes. The presence of phospho-p44/42 was examined by Western blot analysis. Similar patterns were observed in three independent experiments. Results from one experiment are shown. C, human primary stromal cells were treated with the AXIIIR polyclonal antibody for 24 hours. The OPG mRNA expression was examined by RT-PCR. Similar results were seen in two independent experiments. D, Human primary marrow stromal cells were treated with the AXIIIR polyclonal antibody for 48 hours. RANKL and OPG expression levels were detected by Western blot analysis. E, OCL formation assays were performed as described. Goat anti-human OPG neutralizing antibody was added to selected treatment groups. RANKL 30 ng/ml was used as a positive control. Controls for reagents used in these experiments are shown at the left side of the graph. A similar pattern of results was seen in three independent experiments. Results from one experiment are shown and presented as the mean ± SEM. * < 0.05 compared to controls.
Fig. 1

A. Bio-AXIIa + excess AXIIa  
   Bio-AXIIa

B.  

C.  

D.  

E. Kd = 5.79 nM  
   Bmax = 2.13 x 10^9/cell
Fig. 2

A. Biotinylated p36 control

IB: streptavidin

B. Bio-AXIIr

IB: anti-p36

IB: anti-p11

C. ib: streptavidin

IB: Flag

D. hBM MG63 SAKA PSV 10

E. Bound AXIIr (fmol)

Free AXIIr (nM)

Kd = 6.3 nM

Bmax = 7.661

Specific binding
Fig. 3

A.

Chromosome #

B.

a, human
b, hamster
c, mouse
d, rat

C.

<table>
<thead>
<tr>
<th>Resting cells</th>
<th>Activated</th>
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<tbody>
<tr>
<td>mono CD4</td>
<td>CD8 CD14 CD19 mono CD4 CD8 CD19</td>
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<td></td>
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</tbody>
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Spleen
Lymph node
Thymus
Tonsil
BM
Fetal liver
Leukocyte

AX-II receptor
GAPDH

AX-II receptor
GAPDH

Heart
Brain
Lung
Liver
Kidney
Pancreas
Intestine
Colon
Prostate
Testis
Ovary
Skeletal muscle
Placenta
Cloning and characterization of the Annexin II receptor on human marrow stromal cells
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