CD38/ADP-Ribosyl Cyclase: A New Role in the Regulation of Osteoclastic Bone Resorption

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Abstract. The multifunctional ADP-ribosyl cyclase, CD38, catalyzes the cyclization of NAD$^+$ to cyclic ADP-ribose (cADPr). The latter gates Ca$^{2+}$ release through microsomal membrane-resident ryanodine receptors (RyRs). We first cloned and sequenced full-length CD38 cDNA from a rabbit osteoclast cDNA library. The predicted amino acid sequence displayed 59, 59, and 50% similarity, respectively, to the mouse, rat, and human CD38. In situ RT-PCR revealed intense cytoplasmic staining of osteoclasts, confirming CD38 mRNA expression. Both confocal microscopy and Western blotting confirmed the plasma membrane localization of the CD38 protein. The ADP-ribosyl cyclase activity of osteoclastic CD38 was next demonstrated by its ability to cyclize the NAD$^+$ surrogate, NGD$^+$, to its fluorescent derivative cGDP-ribose. We then examined the effects of CD38 on osteoclast function. CD38 activation by an agonist antibody (A10) in the presence of substrate (NAD$^+$) triggered a cytosolic Ca$^{2+}$ signal. Both ryanodine receptor modulators, ryanodine, and caffeine, markedly attenuated this cytosolic Ca$^{2+}$ change. Furthermore, the anti-CD38 agonist antibody expectedly inhibited bone resorption in the pit assay and elevated interleukin-6 (IL-6) secretion. IL-6, in turn, enhanced CD38 mRNA expression. Taken together, the results provide compelling evidence for a new role for CD38/ADP-ribosyl cyclase in the control of bone resorption, most likely exerted via cADPr.

Key words: Ca$^{2+}$ channel • ryanodine receptor • bone resorption • cADPr • osteoporosis

CD38/A DP-ribosyl cyclase is a key cellular enzyme that catalyses the cyclization of the intermediary metabolite, nicotinamide adenine dinucleotide (NAD$^+$), to the putative second messenger, cyclic ADP-ribose (cADPr)$^1$. The latter gates Ca$^{2+}$ release from RyR-gated Ca$^{2+}$ stores (Lee et al., 1994; DeFlora et al., 1998). CD38 is widely distributed in hemopoietic cells, including B and T lymphocytes, thymocytes, plasma cells, macrophages, and erythrocytes, as well as in kidney, car-

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1. Abbreviations used in this paper: BCIP, 5-bromo-4-chloro-3-indoyl-phosphate; cADPr, cyclic ADP-ribose; IL-6, interleukin 6; NBT, 4-nitroblue tetrazolium chloride; RyRs, ryanodine receptors; TRAP, tartrate-resistant acid phosphatase.

diac, pancreatic, brain, spleen, lung, and liver cells (Malavasi et al., 1992; Lee et al., 1996, 1997; Shubinski and Schlesinger, 1997; Fernandez et al., 1998). Structurally, CD38 is a monomeric, 46-kD, type II glycoprotein with a short NH$_2$-terminal cytoplasmic domain, a single membrane-spanning region, and a long extracellular COOH-terminal catalytic domain (Lee et al., 1994). The cDNA’s encoding human, mouse, and rat CD38 have been cloned. The deduced murine and rat CD38 sequences display ~75% homology with the human sequence (Harada et al., 1993; Mehta et al., 1996; Ferrero and Malavasi, 1997). A part from being an ADP-ribosyl cyclase, CD38 can function as a NAD$^+$ glycohydrolase and an ADP-ribose hydrolase (Lee et al., 1994; Berthelier et al., 1998). Intracellularly generated cADPr is thought to play the role of a cellular second messenger (for review, see Lee, 1996; Guse et al., 1999). It is also considered a receptor for CD38 in B and T lymphocytes (Daglio et al., 1998; Horenstein et al., 1998).
There is further evidence that plasma membrane CD 38 internalizes upon binding to monoclonal antibodies (Funaro et al., 1998) and NADP+ (Zocchi et al., 1999). The latter would yield intracellular cADPr that could potentially activate microsomal membrane RyR (Zocchi et al., 1999). A Iso of note is that NADP+, being an intermediary metabolite, could couple a cell’s metabolic activity to its Ca2+ level via the CD 38/cADPr pathway. It is now well established that the osteoclast, a cell that is unique in its ability to resorb bone, can monitor changes in its ambient Ca2+ level by means of a Ca2+ sensor (M algaroli et al., 1989; Zaidi et al., 1989, 1993; M oonga et al., 1990). A high extracellular Ca2+, through a rise in cytosolic Ca2+, triggers dramatic osteoclast retraction, and in the longer term, a marked inhibition of acid secretion, enzyme release, and bone resorption (M algaroli et al., 1989; Zaidi et al., 1989, 1990; M iyauchi et al., 1990; M oonga et al., 1990). We believe that a type 2 ryanodine receptor (RyR-2), positioned uniquely in the osteoclast plasma membrane, functions as a Ca2+ channel, and possibly a Ca2+ sensor (Zaidi et al., 1995). Ordinarily, RyRs are located in microsomal membranes and gate Ca2+ release in response to both Ca2+ and cADPr. Ca2+ sensing in the osteoclast is regulated by several systemic and local factors, namely calcitonin, interleukin-6 (IL-6), ambient pH., and membrane potential (Zaidi et al., 1996; A debanjo et al., 1994, 1998; Shankar et al., 1995). We have shown recently that IL-6 attenuates the inhibitory effect of Ca2+ on the osteoclast (A debanjo et al., 1998). Ca2+ in turn enhances IL-6 secretion, possibly as part of a feedback signal to maintain resorption even in the face of a rising Ca2+ (A debanjo et al., 1998). Of note is that, during resorption, an osteoclast’s metabolic requirements and, hence, its NADP+ levels, are likely to increase dramatically because of active proton and enzyme secretion.

This study examines whether CD 38/A DP ribosyl cyclase has a new role in the regulation of osteoclastic bone resorption. We first report the cloning and sequencing of cDNA encoding a novel CD 38 homologue. Furthermore, we demonstrate that CD 38 mRNA is expressed in the osteoclast; that immunoreactive CD 38 is localized to the cell’s plasma membrane; that the enzyme displays A DP ribosyl cyclase activity in the N G D → cADPr assay; that, when activated, CD 38 triggers a cytosolic Ca2+ signal through ryanodine receptor activation; and that the CD 38-induced Ca2+ signal is associated with resorption inhibition and IL-6 release. We postulate that NADP+ couples an osteoclast’s metabolic activity to its responsive function using CD 38 and cADPr as the sensor and signal, respectively.

Materials and Methods

Osteoclast Cultures

Long bones obtained from neonatal Wistar rats killed by decapitation were cut into fragments and implanted into H epes-buffered M edium 199 containing Hank’s salts (GIBCO-BRL) and heat-inactivated fetal bovine serum (FBS, 5%, vol/vol), and Sigma Chemical Co. (M 199 H). The resulting suspension was dispersed onto denuded cortical bone slices or 22-mm, 0-grade, glass coverslips (LabPro/ICN). Osteoclasts attached to the respective substrate within 15 min (37°C) and contaminating cells were removed by gentle rinsing. Osteoclasts were identified readily by their large size, multinuclearity, complex morphology, densely refracting edges, and response to calcitonin (Zaidi et al., 1992).

Purified rabbit osteoclasts were prepared by the method of K akudo et al. (1996) from unfractionated bone cells obtained according to the procedure described by T ezuka et al. (1992). In brief, cell suspensions obtained from minced long bones of 10-d-old rabbits (Japan White, Salamta X erimental A nimal Supply Co.) were agitated by vortexing and plated in 10-cm tissue culture dishes (Becton Dickinson) coated with 2% collagen gel (Nitta Gelatin Co.). A 3-h incubation period, adherent non-osteoclasts were removed from the collagen gel by sequential treatment with pronase E (0.001% wt/vol) and collagenase (0.01%, wt/vol), Wako Pure Chemical Industries. The remaining osteoclasts were then collected by 0.1% (vol/vol) collagenase solution treatment and replated. When these cell suspensions were seeded onto tissue culture dishes, osteoclasts attached and spread out. The purity of the osteoclast preparation was judged before membrane isolation by staining for an osteoclast-specific marker, tartrate-resistant acid phosphatase (TRAP) using a leukocyte acid phosphatase kit (Sigma). In line with previous experiments of Kameda et al. (1997), we found that the purity of TRAP-positive multinucleated cells (>3 nuclei) was >99%. These cells have been shown to resorb bone and express specific osteoclast markers, cathepsin K, and calcitonin receptors (Takeda et al., 1992; Kameda et al., 1997, 1998).

Isolation and Sequencing of CD 38 cDNA Clone from a Rabbit Osteoclast cDNA Library

A rabbit osteoclast cDNA library containing 1 X 109 independent clones was used for PCR amplification (T ezuka et al., 1992; Kameda et al., 1997). Two oligonucleotide primers were designed from the known rat CD 38 cDNA sequence (these data are available from GenBank/EMBL/DDJB under accession D 29646): forward primer, 5′-CCCTGTTGCTGT-GTTCTGGA-3′ (569-588), and reverse primer, 5′-GGTGCTCAGTAGTTACCTGG-3′ (863-884) (GIBCO-BRL). The coding region of rabbit CD 38 cDNA fragment was then amplified by PCR. In brief, the standard reaction mixture (50 μl) contained: 0.1 μl of rabbit osteoclast cDNA library, 1 μl of each oligonucleotide (50 μl), and 1 μl (5 U) of AmpliTaq (Perkin Elmer). The PCR products were separated by agarose gel electrophoresis. A ~300-bp DNA fragment was isolated from excised gel slices using a QIAquick Gel Extraction Kit (QIAGEN Inc.) and ligated into EcoR V-cut pBluescript II SK (+) (Stratagene) to produce independent clones. Sequencing of the inserts was performed (15-20 bp) using an Applied Biosystems 377 instrument. For this, the probe was labeled with α-[3P]CTP (3000 Ci/mmold (NE 2 L Life Science Products Inc.) using the RedPrime Random Prime Labeling Kit (A mersham Pharmacia Biotech Inc.). Duplicate filters, which covered 1 X 109 independent clones, were then hybridized overnight at 42°C with prehybridization solution (50% formamide, 6× SSC, 5× Denhardt’s, 0.5% SDS, 0.1 mg/ml denatured fragmented salmon sperm DNA) to which a labeled probe was added. After a 2-h hybridization step at 68°C for 1 h, the filters were washed with x-ray film intensifying screens for 20 h at ~70°C. Positive recombinant plagues were purified from phage plate lysates according to the Lambda ZAP II library’s instruction manual (Stratagene). The DNA clones were confirmed by NsiI-KpnI restriction analysis and direct nucleotide sequencing.

CD 38 mRNA Expression in Single Osteoclasts Revealed by In Situ RT-PCR Cytoimaging

We and others have recently applied this technology to examine CD 38 expression in single rat osteoclasts (primer sequences, as above). To a positive control, we also examined the expression of a housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase, GAPDH, data available from GenBank/EMBL/ DDBJ) under accession number M 32599) and an osteoclast-specific gene (cathepsin K, accession number A F 010306). Their primer sequences were:

GAPDH, forward: 5′-TGAAGTTCGGTGTCATGAGGTT-3′
McArdle, 1978). The cells were then incubated without antibody, or with non-
immune mouse IgG, Ab (anti-CD38 antibody) (1:1,000; Sigma Chemical Co.) and NAD
(1 M NGDPr) then washed gently, and finally, drained. The number of fluorescent osteoclasts
was first determined in a laser confocal scanning microscope, at λex = 458 nm (Ar+) (λem = 525 nm), to locate the osteo-
clast membrane, 1-μm-thick optical sections were obtained in the cell’s coronal plane in selected experiments. Finally, trypan blue (1 mM, 961 Da; Sigma Chemical Co.) was applied to exclude membrane damage that could allow antibody access into the cell.

Membrane Isolation and Western Blot Analysis
For isolation of plasma membranes, cells were first scraped and homogen-
ized in TKM solution (50 mM Tris-HCl, pH 7.5, 25 mM KCl and 5 mM MgCl2) supplemented with 0.25 mM sucrose. The homogenate was centrif-
uged (3,000 g, 10 min), the pellet resuspended in sucrose (70% wt/vol), and then rehomogenized (12 strokes) with a glass/Teflon homogenizer. The sucrose solution was then layered as follows: the homogenate was overlaid with 12 ml of 48% (wt/vol) sucrose, followed by 6 ml of 42% (wt/ vol) sucrose. This was then centrifuged at 27,700 rpm for 70 min in a SW-
28 swinging bucket rotor. The plasma membrane fraction banding at the interface of 70%/48% sucrose was collected and suspended in 70% (wt/ vol) sucrose solution. The entire process was repeated twice to purify the plasma membranes.

SDS-PAGE was performed using 12% separating and 4% stacking polyacrylamide gels using a minigel system (BioRad Laboratories). Plasma membranes prepared from osteoclasts and osteoblasts (30 μg protein) were heated for 5 min at 95°C in Laemli’s sample buffer (2% SDS, 2% mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue in 0.1 M Tris-HCl buffer, pH 6.8). Electrophoresis was performed at 20 mAmps per gel. The proteins thus resolved were stained with Coomassie Brilliant Blue (Sigma Chemical Co.) or transferred electrophoretically onto OPTITRAN-supported nitrocellulose membrane (Schleicher and Schuell) at 15°C for 1 h at 100 volts. The membranes were blocked with Tween 20 (0.3% vol/vol) in PBS at 20°C and incubated with the anti-
CD38 antibody (1:3,000) (Sigma Chemical Co.). A fer rinsing, the blot was incubated for 1 h with HRP-conjugated anti-mouse antibody. The blot was developed using Pierce SuperSignal Ultra Chemiluminescence Kit, per manufacturer’s instructions.

ADP-Ribosyl Cyclase (NGD + → eGDPPr) Assay
A D-ribosyl-cyclase activity was measured in osteoclast plasma membranes isolated as above. We measured the cyclization of the NAD+ surro-
gate, NGD+, to its fluorescent derivative, eGDPPr. Plasma membranes (25 μg) were incubated, for 20 min at 37°C, in 20 mM Tris-HCl (pH 7.4) with 100 μM NGD+. The reaction was stopped with 5% (vol/vol) trichloroacetic acid. Fluorescence in the supernatant was measured using a high-sensitivity spectrophuorometer (λex = 390 nm; λem = 410 nm). The amount of cGDPPr formed was plotted as mean ± SD in nmol/ml/mg protein. To establish specificity of the assay, we incubated membranes in with anti-CD38 antibody (1:1,000; Sigma Chemical Co.) and NAD+ (400 μM). Mouse IgG5 was used as control.

Measurement of Cytosolic Ca2+ in Single Osteoclasts
Glass coverslips containing freshly isolated osteoclasts were incubated in serum-free medium (30 min, 37°C) with 10 μM fura 2JA M (Molecular Probes), then washed in M 199-H and transferred to a Perspex bath posi-
tioned on the microspectrofluorometer stage. The latter was previously con-
structed from an inverted microscope (Diaphot; Nikon) (Shankar et al., 1992). Prewarmed test solutions of the anti-CD38 antibodies (A 10, agonist and antagonist; Sigma Chemical Co.) (1:500), NAD+ (0.5, 1, or 10 mM), ryan-
odine (5 μM), caffeine (250 μM and 1 mM), or thapsigargin (4 μM) were applied in various protocols, as described in Results. The cells were exposed alternatively to excitation λ of 340 or 380 nm. The emitted fluo-
rescence was detected through a 400-nm dichroic mirror and subsequently filtered at 510 nm. The signal was converted to 25 ns, 5V transis-
tor-transistor-logic (TTL) pulses in a photomultiplier tube (PM 28B; Thorn EM1). The resulting pulses were counted by a dual photon counter (Newcastle Photometrics) and recorded every second to give a ratio of emitted intensities at excitation λ of 340 and 380 nm, R(340-380).

The cytosolic Ca2+ measuring system was calibrated using an estab-
lished protocol for intracellular calibration (Shankar et al., 1993). In brief, fura 2-loaded osteoclasts were bathed in Ca2+-free, EGTA-containing so-
lution containing 130 mM NaCl, 5 mM KCl, 5 mM glucose, 0.8 mM MgCl2, 10 mM HEPES, and 0.1 mM EGTA. 5 μM ionomycin was first applied to obtain the minimum ratio due to lowest cytosolic Ca2+ (Rs0) and the

Antibodies
Dr. F. Malava (Torino, Italy) kindly provided the monoclonal anti-CD38 antibody, A 10. A 10 was raised by immunizing mice with B urtic. lympho-
a. D cells using the monkey lympho-
ma cells (M alava et al., 1984, 1985). The antibody recognizes a 46-kD CD38 molecule on T cells and B lymphocytes also enhancing their acti-
ation and proliferation (hence the term, agonist) (Furuno et al., 1990). A nega-
tant antibody (Sigma Chemical Co.) was also used to establish specificity for CD38 detection in the ADP ribosyl cyclase assay. The con-
trol anti-rhodamine receptor antibody, A b, was raised against a cytosolic calmodulin-binding RyR epitope. Therefore, it does not stain nonperme-
abilized osteoclasts (Zaidi et al., 1995).

Immunocytochemistry and Confocal Microscopic Analysis
Osteoclasts were incubated with normal goat serum (in 10 mM PBS, 1:10, pH 7.4, 35 min) in multwell dishes and washed with HBSS (G1BCO-
BR.) L cells were also incubated without antibody, or with non-
immune mouse IgG, A b (anti-RyR antibody) (all controls), or A 10 (anti-CD38 antibody) (in M 199-H, 1:100). In the same experiment, CD38-
negative fibroblasts were also incubated with the same antibodies. The coverslips were rinsed gently with HBSS, drained, reincubated with goat anti-mouse FITC (Sigma Chemical Co.; 1:100, in HBSS, 60 min),
maximum fluorescence intensity at 380 nm (F_max). 1 mM CaCl_2 was then applied with 5 μM ionomycin to obtain values of the maximum ratio due to an elevated cytosolic Ca^{2+} ([Ca^{2+}]_c) and the minimum fluorescence intensity at 380 nm (F_min). The dissociation constant K_d for Ca^{2+} and fura 2 is 224 nM (20°C, 0.1 M, pH 6.85). The values were substituted into the equation: [Ca^{2+}]_c = K_d \times (R - R_{max})/(R_{max} - R) \times (F_{max}/F_{min}). Mean changes (Δ) in the cytosolic Ca^{2+} concentration ([Ca^{2+}]_c) were then calculated by subtracting peak from basal cytosolic [Ca^{2+}]. Statistical comparisons of cytosolic [Ca^{2+}]_c were made by Analysis of Variance (ANOVA) with Bonferroni’s Correction for Inequality.

### Bone Resorption Assay

Bone resorption was measured using the pit assay (Boyd et al., 1984; Chambers et al., 1984; Dempster et al., 1987). In brief, the bones from 24- to 48-h-old rats were sliced in 3.5 ml M 199-H, and the resulting cell suspension was settled onto devitalized cortical bone slices (4 mm × 4 mm) for 30 min. A fter the removal of nonadherent cells by gentle rinsing, the slices were transferred to a multwell dish containing M 199-H (with 10% FBS vol/vol). Either vehicle or anti-CD38 antibody (1:500) and NAD (1 mM). The culture medium was changed, and the slice was incubated for 24 h in humidified CO_2 (5%) (pH 6.9), after which they were fixed with glutaraldehyde (10% vol/vol) and stained for the presence of tartrate-resistant acid phosphatase (TRAP) using a kit (Kit 386A; Sigma Chemical Co.). The number of osteoclasts with two or more nuclei was determined on each slice using light microscope (Olympus). The cells were removed by treating the slices with NaOCl (5 min), and the slices rinsed with distilled water followed by acetone, and then air-dried. The slices were stained subsequently with toluidine blue (1% vol/vol, in 1% wt/vol borate, 5 min). The number of resorption pits was determined on each slice by light microscopy. Notably, each experiment was performed with osteoclasts obtained from three animals with five or six bone slices per treatment. The number of pits or osteoclasts per bone slice was expressed as a mean ± SEM. Student’s unpaired t-test was used to analyze the effect of treatment, which was considered significant at P < 0.05.

### Supernatant IL-6 Measurements by ELISA

Osteoclasts on coverslips were bathed in a multwell dish containing 50 μl M 199-E (with 10% FBS vol/vol) for 6 h in the presence of either vehicle or anti-CD 38 antibody (1:500) and N A D^- (1 mM). The culture medium was changed, and the IL-6 level was measured with an ELISA kit (M 19900; R&D). In brief, 96-well plates coated with a polyclonal anti-mouse IL-6 antiserum were used to accommodate 50 μl of assay diluent (buffered protein) and 50 μl of standard, control, or sample. A fter incubation (20°C, 2 h), the wells were aspirated, washed repeatedly, and loaded with 100 μl horseradish peroxidase–conjugated anti-IL-6 antibody. A fter a further incubation (20°C, 2 h), 100 μl of substrate solution containing H_2O_2 and tetramethylbenzidine, was added to each well. Finally, a further incubation (30 min) was followed by the addition of 100 μl of dilute HCl to stop the reaction. The optical density of each sample was measured at 450 nm on a microplate reader (Bio-Rad). IL-6 was estimated from the standard curve in triplicate experiments and represented as mean ± SEM. Differences between control and treatment were assessed by the Student’s unpaired t-test.

### Results

#### Isolation and DNA Sequence Analysis of a 2.8-kb Rabbit Osteoclast CD38 cDNA

To obtain full-length CD 38 cDNA clones, a rabbit osteoclast cDNA library was screened. A 293-bp CD 38 cDNA coding region DNA fragment was initially cloned and used as probe. A single positive cDNA clone was identified after screening 1 × 10^7 independent phage recombinants; this contained a 2.8-kb EcoRI-XhoI insert in the plasmid pBluescript-SK (termed SL 385). The sequence of the full-length SL 385 CD 38 insert was obtained. Sequence analysis confirmed the presence of the CD 38 coding sequence and extended into 3'-untranslated region (Fig. 1). The osteoclast CD 38 cDNA sequence was 71, 69, and 66% similar to corresponding full-length CD 38 cDNA sequences of mouse, rat, and human CD 38 (obtained from the GenBank database) (Fig. 1). No significant homology was found, however, between the sequence of the insert and any other sequence in the GenBank database. Fig. 2 shows the predicted amino sequence of the full-length rabbit osteoclastic CD 38. There was a 59, 59, and 50% similarity between this sequence and that of mouse, rat, and human CD 38 (GenBank, respectively). The relative sequence divergence suggests that the amplified DNA product codes for a yet uncharacterized member of the CD 38 family of cyclases.

### CD38 mRNA in Single Osteoclasts Demonstrated by In Situ RT-bPCR Cytomaging

CD 38 mRNA expression in isolated single osteoclasts was investigated by in situ RT-PCR using the same primers as used for PCR cloning (above). Fig. 3 shows light micrographs of histostained osteoclasts after RT-PCR. Panel i shows an unstained osteoclast (negative control) in an experiment in which primers were omitted from the reaction mixture. Panels ii and iii show osteoclasts in which the intense bluish-brown staining represents, respectively, mRNA expression for cathepsin K (cell-specific positive control) or GAPDH (housekeeping gene). Panels iv to vi show intense CD 38 mRNA histostaining in osteoclasts that were either incubated with vehicle (iv), 10 ng/liter IL-6 (v), or 10 μg/liter IL-6 (vi).

Fig. 4 shows a semi-quantitative analysis of staining intensity using a method modified from that reported by Adebajo et al. (1998). Osteoclasts staining for CD 38 mRNA were thus assessed by a blinded observer who assigned an intensity level to the staining as a number between 0 and 4 (no staining to intense staining). Three experiments were pooled to derive frequency histograms relating the number of cells to their assigned intensity score. Osteoclasts that underwent in situ RT-PCR without added primers (control) showed a skewed distribution to the left (i, n = 26 cells). Cells incubated with primers, but without treatment for 6 h with IL-6 (ii, n = 49 cells) or those treated with 10 ng/liter IL-6 (iii, n = 53 cells), showed a normal (Gaussian) distribution of their assigned scores. The data became significantly skewed (P < 0.05) to the right when osteoclasts were treated with 10 μg/liter IL-6 (iv, n = 29 cells). In contrast, the expression of mRNA for GAPDH, the housekeeping gene, followed a similar distribution in all three experimental sets, namely no treatment, 10 ng/liter IL-6, and 10 μg/liter IL-6 (not shown). Taken together, the results showed that a much larger proportion of cells stained intensely with 10 μg/liter IL-6 compared with 10 ng/liter IL-6, suggesting that the greater concentration of IL-6 might enhance CD 38 mRNA levels. As in our earlier study (Adebajo et al., 1998), we must emphasize that the results are semi-quantitative at best, due not only to the inherent pitfalls of the in situ RT-PCR technique, but also because the cells may undergo slight margin retraction resulting in scoring artifacts. A gain, as before, we have excluded obviously retracted cells, as in these cells, staining is likely to appear more intense due to cytoplasmic condensation.
Figure 1. Nucleotide sequence of rabbit osteoclast CD38 cDNA compared with the known mouse, rat, and human sequences, as shown. The respective sequences were 59, 59, and 50% homologous with the rabbit CD38 sequence. The 5′- and 3′-untranslated regions (−76 to 21 bp and 2,798 to 898 bp, respectively) are also shown. The start and stop codons are indicated in bold. Gaps are introduced to maximize homology.
that Ab34 was raised against a cytosolic calmodulin-binding treatment provided clear evidence for specificity. Note (Fig. 5 A). That osteoclasts did not stain with any such using an irrelevant anti-ryanodine receptor antibody, Ab34 immune mouse IgG instead of the antibody (not shown); (c) erwise permit antibody access into the cytosol.

trypan blue, excluding membrane damage that would oth-

tive staining. Furthermore, all cells remained negative for clasts examined in each different experiment showed posi-

blest were found not to stain with the antibody (not shown). Also of note is that every one of the classtains with the antibody (not shown).

Cloning and Expression of CD38

We next examined whether our highly specific anti-CD38 antibody, A10, bound to the surface of intact live osteoclasts (not shown). From postnuclear membranes prepared MC3T3-E1 osteoblasts (not shown).

ADP Ribosyl Cyclase Activity in Osteoclast Plasma Membranes in the NGD \(^{-}\) \(\rightleftharpoons\) cGDPr Assay

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We further confirmed that the CD38 protein was pres-

Cytosolic Ca\(^{2+}\) Signals Triggered through CD38 Activation and cADPr Generation

Having established the presence of CD38 in the osteoclast plasma membrane, we next investigated the effects of its activation by the agonist anti-CD38 antibody. Thus, we measured changes in cytosolic [Ca\(^{2+}\)] in response to application of NA D\(^{+}\) (substrate) in the presence of the agonist antibody. Expectedly, only in the presence of the antibody (1,500), did 1 mM NA D\(^{+}\) trigger a cytosolic [Ca\(^{2+}\)] elevation (Fig. 8 A). This result was consistent with an activated CD38/A D-ribose cyclase that catalyzes cA DPr generation from NA D\(^{+}\). In separate experiments, the anti-CD38 antibody itself, in the absence of NA D\(^{+}\), did not elevate cytosolic [Ca\(^{2+}\)], indicating that the substrate, NA D\(^{+}\), was necessary for CD38-induced Ca\(^{2+}\) signaling (not shown). Finally, 1 mM NA D\(^{+}\) failed to trigger a cytosolic Ca\(^{2+}\) signa in the presence of the control anti-RYR antibody, A B34, further confirming response specificity.

A t higher, 10 mM, NA D\(^{+}\) concentrations, a marked elevation in cytosolic [Ca\(^{2+}\)] was noted even in the absence of the antibody (Fig. 8 A). This response was significantly different (P = 0.013) to the control response (1 mM NA D\(^{+}\) alone), but did not differ significantly (P = 0.22)
from the response triggered by 1 mM NAD$^+$ with antibody (Fig. 8 A).

We further demonstrated CD38-specificity of the NAD$^+$-induced cytosolic Ca$^{2+}$ response by preincubating osteoclasts with the anti-CD38 antagonist antibody (Sigma Chemical Co.) before application of 10 mM NAD$^+$. The antagonist antibody attenuated the magnitude of the cytosolic Ca$^{2+}$ response significantly (Fig. 8 C).

To determine whether NAD$^+$ triggered the release of Ca$^{2+}$ from intracellular stores, we carried out experiments with 10 mM NAD$^+$ in the presence or absence of 2 mM EGTA (to chelate extracellular Ca$^{2+}$ to near-nanomolar levels) or thapsigargin (a microsomal membrane Ca$^{2+}$-ATPase inhibitor that is known to deplete intracellular Ca$^{2+}$ stores). The response to 10 mM NAD$^+$ in Ca$^{2+}$-free, EGTA-containing medium remained unchanged compared with that to 10 mM NAD$^+$ in 1.25 mM Ca$^{2+}$ ($P = 0.335$). Furthermore, Fig. 8 B shows that when cells were treated with 4 μM thapsigargin, there was a significant attenuation of the cytosolic Ca$^{2+}$ response to 10 mM NAD$^+$. However, it is notable that thapsigargin did not completely abolish the cytosolic Ca$^{2+}$ response to NAD$^+$ suggesting that the Ca$^{2+}$ signal was not completely dependent upon the fullness of intracellular Ca$^{2+}$ stores. Taken together, the results suggested that NAD$^+$ primarily triggered the release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores, although Ca$^{2+}$ influx may also play a role.

We next attempted to test the hypothesis that NAD$^+$-induced cADPr generation resulted in the activation of intracellular ryanodine receptors. For this, we examined whether the known cell permeant ryanodine receptor

Figure 3. Histostained osteoclasts following in situ RT-PCR for detection of CD38 mRNA. i shows a negative control from a representative experiment i.e., without added primer. ii and iii represent histostaining for cathepsin K (Cath K) (cell-specific positive control) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (housekeeping gene). Panels iv to vi show CD38 mRNA staining in osteoclasts incubated either in vehicle (iv) or with 10 ng/liter IL6 (v) or 10 μg/liter IL6 (vi). For details and primer sequences refer to Materials and Methods.

Figure 4. Semiquantitative representation in frequency histograms of intensity score after in situ RT-PCR for CD38 mRNA in osteoclasts incubated either without primer (i) or with CD38 primers without IL-6 treatment (ii) or with IL-6 treatment (10 ng/l, iii, or 10 μg/l, iv). Staining intensity was graded as described in Materials and Methods by an independent blinded observer who scored the intensity from zero (no staining) to 4 (intense staining) in three experiments. The data were analyzed statistically for skewness and shifts were considered significant if $P < 0.05$. 
modulators, ryanodine and caffeine, inhibited the response to applied NAD$^+$. Both ryanodine (5 μM) and caffeine (250 μM and 1 mM) significantly inhibited the cytosolic Ca$^{2+}$ response to NAD$^+$ (P values, see legend to Fig. 8). Taken together, the results suggest that RyR-gated Ca$^{2+}$ stores were being emptied in response to NAD$^+$, implicating, though not proving, a direct role of cADPr as a second messenger. This is consistent with our direct demonstration of cADPr-forming, A DP-ribosyl cyclase activity in the osteoclast plasma membrane as assessed by the NGD→cGDPPr assay (Fig. 7). It should be emphasized that thapsigargin, ryanodine, and caffeine have all been used as tools to understand the mechanism of NAD$^+$-induced Ca$^{2+}$ signaling, and in view of their other cellular actions would not be expected to reverse the effect of NAD$^+$ on bone resorption and IL-6 release.

**Inhibition of Bone Resorption and Enhancement of IL-6 Release by CD38 Activation**

We have shown that while a cytosolic Ca$^{2+}$ change triggers resorption inhibition, it elevates IL-6 synthesis and release (Zaidi et al., 1989, Moonga et al., 1990, Adebanjo et al., 1998). Our goal, therefore, was to examine the effect of CD38 activation by NAD$^+$ (in the presence of its agonist antibody, A10) on bone resorption and IL-6 release. In the presence of A10, at either dilutions (1:5,000 or 1:500), 1 mM NAD$^+$ inhibited osteoclastic bone resorption significantly (P = 0.034 and P = 0.025, respectively, compared with vehicle-treated cells) (Fig. 9 a). Expectedly, osteoclast number per slice did not change significantly (P > 0.05 for either antibody dilution) (Fig. 9 b), excluding an effect of the antibody on osteoclast formation or demise. In separate experiments, the 1 mM NAD$^+$ (in the presence of A10, 1:500), caused a dramatic and highly significant threefold elevation (P < 0.001) of IL-6 release (Fig. 9 c). Taken together, the results appear consistent with the paradoxical effects of Ca$^{2+}$ on bone resorption and IL-6 release (Moonga et al., 1990; Adebanjo et al., 1998).

**Discussion**

The multifunctional ectoenzyme, CD38, is known to modulate lymphocyte functions as critical as adhesion, proliferation and cytokine production (Cesano et al., 1998, Ferrero and Malavasi, 1997). It also functions as a counter-receptor for CD31, presumably facilitating cell-to-cell communication (Deaglio et al., 1998; Horenstein et al., 1998). It is also an A DP-ribosyl cyclase that catalyzes the formation of cADPr from NAD$^+$. Several reports have suggested that the latter is a cellular second messenger, somewhat akin to IP$_3$ (for review: Lee, 1996; Guse et al., 1999). We show that CD38 regulates osteoclastic bone resorption via the production of cADPr. Specifically, we show that a novel CD38 homologue is located in the rabbit osteoclast plasma membrane; that it possesses A DP-ribosyl cyclase activity; that its activation results in cytosolic Ca$^{2+}$ elevation through ryanodine receptor activation; and that the cytosolic Ca$^{2+}$ change is accompanied, quite expectedly, by an elevation in IL-6 release and resorption inhibition.
CD38 catalyzes the cyclization of NAD$^+$ not only to cADPr (Howard et al., 1993), but also to the more recently described, dimeric ADPPr (Delflora et al., 1997a). While the classical action of cADPr is to release Ca$^{2+}$ from RyR-bearing Ca$^{2+}$ stores, dimeric ADPPr potentiates this effect (Delflora et al., 1997a). In the osteoclast, we have shown that cADPr triggers both Ca$^{2+}$ release and Ca$^{2+}$ influx through its action, respectively, on microsomal membrane RyRs and a uniquely positioned surface RyR-2 (Zaidi et al., 1995; Adebajo et al., 1996). A part from being activated by cADPr, the uniquely positioned osteoclast surface RyR-2 appears also to sense changes in the cell’s ambient Ca$^{2+}$ concentration during resorption (Zaidi et al., 1995). Any rise in cytosolic Ca$^{2+}$ in the osteoclast triggers rapid cell retraction, diminished enzyme release, and reduced acid secretion, culminating finally, in the inhibition of bone resorption (M’algaroli et al., 1989; Zaidi et al., 1989; Datta et al., 1990; Miyachi et al., 1990; Moonga et al., 1990). However, an increased cytosolic Ca$^{2+}$ also enhances IL-6 secretion, possibly to release an osteoclast from the resorption inhibition induced by a high Ca$^{2+}$ (Adebajo et al., 1998).

The observed effect of CD38 activation in inhibiting bone resorption and elevating IL-6 release thus mirrors that of Ca$^{2+}$. Notably, both agents act by elevating cytosolic Ca$^{2+}$. Interestingly, however, cADPr-induced Ca$^{2+}$ release also mediates the effect of CD38 in inducing other cytokines, including IL-6, interferon-γ, granulocyte-macrophage colony stimulating factor (GM-CSF), and IL-10 (Ausiello et al., 1996). Cyclic ADPPr also promotes secretion of hormones, such as insulin from pancreatic β cells, and cytokines from T cells (Takasawa et al., 1998; Cesano et al., 1998). Nevertheless, it is unclear as to how these released cytokines, in turn, affect CD38 expression and cADPr formation. We provide new evidence that IL-6 enhances the expression of CD38 mRNA. This appears consistent with a NF-IL-6 site in the CD38 gene promoter (Kishimoto et al., 1998). Our in situ RT-PCR results, however, must be treated with caution in view of the known technological pitfalls and possible artifacts, which we have tried to avoid.

The Ca$^{2+}$-like effects of CD38 might also be relevant physiologically in the metabolic control of bone resorption via NAD$^+$. It is noteworthy that the energy requirement of a resorbing osteoclast is high due to its active secretion of acid and enzymes and its intense motile activity. It is therefore possible that large amounts of NAD$^+$ are being generated intracellularly during resorption. Significant amounts of this NAD$^+$ may indeed extrude from the osteoclast. Indeed, Zocchi et al. (1999) have demonstrated the existence of a saturable and bidirectional NAD$^+$ transport system in a variety of eukaryotic cells; the same could be true for osteoclasts. Alternatively, neighboring cells undergoing apoptosis may release much NAD$^+$ (Meha et al., 1996). The extracellularly located catalytic domain of the CD38/ADP-ribosyl cyclase could then sense the NAD$^+$, and by catalyzing its conversion to cADPr, limit further osteoclastic resorption. Franco et al. (1998) have demon-

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**Figure 8.** Effect of NAD$^+$ (NAD$^+$, concentrations as shown) on the mean change ($\Delta$) in cytosolic [Ca$^{2+}$] (nM) in fura 2-loaded single osteoclasts under various experimental conditions. (A) Osteoclasts were pretreated for 15 min with thapsigargin (4 μM), or caffeine (4 μM). (B) Osteoclasts were pretreated for 15 min with the antagonist antibody (Sigma Chemical Co.) (1:500) (Sig). Cytosolic [Ca$^{2+}$] was calculated in each case by subtracting the basal from peak cytosolic [Ca$^{2+}$]. A asterisks indicate P < 0.05 (n = 6 per group).

**Figure 9.** Effect of the agonist anti-CD38 antibody (A 10, 1,500, or 1,500) in the presence of substrate (1 mM NAD$^+$) on bone resorption (a, pits per slice) and osteoclast number (b, osteoclasts per slice) as assessed in the pit assay, as well as on supernatant interleukin-6 (IL-6, ng/liter) levels (c) measured by ELISA. P values as indicated (n = 10 slices per group).

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shown a role of CD 38 in NAD \(^+\) sensing in HeLa cells and human erythrocytes.

Our evidence for the production of cADPr through NAD \(^+\) catalysis by CD 38 is twofold. First, we have directly demonstrated that osteoclast plasma membranes that are positive for CD 38 immunoreactivity contain A DP-riboosyl cyclase activity. This has been assessed using an assay that allows for the catalytic conversion of the NAD \(^+\) surrogate, NGD \(^+\), to its nonhydrolyzable and fluorescent derivative, cGDPPr. We showed that the observed A DP-riboosyl cyclase activity could be inhibited noncompetitively by an antagonist antibody to CD 38, confirming directly, a role for CD 38 in cGDPPr formation. That NAD \(^+\) also significantly inhibited NGD \(^+\) catalysis confirmed further that the two molecules most likely shared the same substrate-binding site. cGDPPr formation in osteoclast plasma membranes thus appears truly reflective of the A DP-riboosyl cyclase activity of CD 38. Second, and in line with the above, we have shown that NAD \(^+\) application to osteoclasts triggers cytosolic Ca\(^{2+}\) release mostly from intracellular stores that are sensitive to inhibition by RyR modulators, ryanodine and caffeine. This, albeit indirect, demonstration for a role of RyRs. Effects of extracellularly applied cADPr on cellular influx. This, however, remains to be established.

Despite our molecular and biochemical demonstration of functionally active CD 38/A DP ribosyl cyclase in the osteoclast plasma membrane, it remains unclear how any A DP Pr synthesized extracellularly could act on intracellular RyRs. Two explanations have been offered in other models (Lund et al., 1998). First, the CD 38 catalytic compartment may become internalized after its recognition of substrate, thus generating A DP Pr intracellularly (Funaro et al., 1998; Zocchi et al., 1998, 1999). In fact, Zocchi et al. (1999) have shown, using endocytic vesicles, that NAD \(^+\) first internalizes through a saturable transport system independent of CD 38, and once, within the vesicle, is catalyzed to cADPr. The latter is then pumped out into the cytosol to affect Ca\(^{2+}\) release from RyR-gated Ca\(^{2+}\) stores. It has been suggested that agonist antibodies, such as A 10, may aid such internalization (Funaro et al., 1998). Indeed, A 10 is known to enhance the activation and proliferation of B and T lymphocytes through enhanced A DP Pr production (hence the term, agonist) (Funaro et al., 1990). Such a mechanism provides one likely explanation for the synergistic effects of the A 10 and NAD \(^+\) on cytosolic Ca\(^{2+}\). An alternative possibility, however, also exists. This is that A DP Pr is first generated extracellularly, and then traverses the cell membrane to interact with intracellular RyRs. Effects of extracellularly applied A DP Pr on cellular function have been described in rat cerebellar cells (De-Flora et al., 1996, 1997b), murine B lymphocytes (Howard et al., 1993) and rat osteoclasts (A debanjo et al., 1996).

Franco et al. (1998) have shown, however, using human erythrocyte membranes and CD 38-reconstituted proteoliposomes that CD 38 is a selective transporter of catecholglycerol and caffeine. This, however, major goal of this study has not been to probe this mechanism; instead, it has been to identify a plausible role of CD 38/A DP ribosyl cyclase in the control of osteoclastic bone resorption.

We have provided evidence that the NAD \(^+\)-induced Ca\(^{2+}\) signal is made up of two components, Ca\(^{2+}\) release from RyR-gated intracellular stores, and Ca\(^{2+}\) influx possibly through the uniquely positioned plasma membrane RyR-2. The role of ryanodine receptors has been generally confirmed through experiments demonstrating that the cytosolic Ca\(^{2+}\) response to NAD \(^+\) is inhibited strongly by both ryanodine and caffeine (Fig. 8). However, these experiments have not allowed us to determine whether the respective modulators block the intracellular RyRs, or the surface RyR-2, or both. Nonetheless, we show here that the NAD \(^+\)-induced cytosolic Ca\(^{2+}\) response is maintained in Ca\(^{2+}\)-free, E G T A -containing medium, suggesting its dependence on intracellular Ca\(^{2+}\) release. Our experiments with thapsigargin, a microsomal membrane Ca\(^{2+}\)-A TPase inhibitor known to deplete intracellular Ca\(^{2+}\) stores, appear more conclusive. These results show that thapsigargin attenuates, but does not abolish the cytosolic Ca\(^{2+}\) signal, suggesting that there is a component of extracellular Ca\(^{2+}\) influx. This, however, remains to be established.

In conclusion, we have documented a new function for osteoclastic CD 38. We believe that its activation at the osteoclast plasma membrane results in cytosolic Ca\(^{2+}\) release from RyR-gated intracellular Ca\(^{2+}\) stores via A DP Pr generation from NAD \(^+\). The released Ca\(^{2+}\) then signals a reduction in bone resorption and a paradoxical elevation of IL-6 release. It is therefore possible that the CD 38/Ca\(^{2+}\)/ IL-6 pathway may have a critical role in coupling an osteoclast's metabolic activity with its resorptive function. Our current studies with CD 38−/− mice should shed more light on the function of CD 38 in osteoclast control (Kato et al., 1999).

The authors are grateful to Professor Iain M Mcntyre (William Harvey Research Institute, London, U K ) for his encouragement and support; Christopher L.-H. Hu (Physiological Laboratory, Cambridge, U K ) for helpful discussion; Qinwu Lin (Wistar Institute, Philadelphia, PA ) for assistance with confocal microscopy; Jerry Rosenzweig (Geriatrics Department, Veterans Affairs Medical Center, Philadelphia, PA ) for assistance in grant management; and Stacey Marshall (University of Pennsylvania, Philadelphia, PA ) for illustrations.

M. Zaidi acknowledges the support of the National Institutes of Health (RO1-AG14702-01) and the Department of Veteran's Affairs.

Submitted: 8 October 1998
Revised: 14 July 1999
Accepted: 26 July 1999

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