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SH3BP2 is an activator of NFAT activity and osteoclastogenesis

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ABSTRACT

Heterozygous activating mutations in exon 9 of *SH3BP2* have been found in most patients with cherubism, an unusual genetic syndrome characterized by excessive remodeling of the mandible and maxilla due to spontaneous and excessive osteoclastic bone resorption. Osteoclasts differentiate after binding of sRANKL to RANK induces a number of downstream signaling effects, including activation of the calcineurin/NFAT (nuclear factor of activated T cells) pathway. Here, we have investigated the functional significance of SH3BP2 protein on osteoclastogenesis in the presence of sRANKL. Our results indicate that SH3BP2 both increases nuclear NFATc1 in sRANKL treated RAW 264.7 preosteoclast cells and enhances expression of tartrate resistant acid phosphatase (TRAP), a specific marker of osteoclast differentiation. Moreover, overexpression of SH3BP2 in RAW 264.7 cells potentiates sRANKL-stimulated phosphorylation of PLCγ1 and 2, thus providing a mechanistic pathway for the rapid translocation of NFATc1 into the nucleus and increased osteoclastogenesis in cherubism.

Cherubism (OMIM 118400) is an autosomal dominant disorder characterized by extensive bone remodeling within the mandible and/or maxilla. The onset of cherubism is early, age 2–5 years, at which time patients develop characteristic giant cell lesions in the maxilla and mandible and substantial facial swelling and cervical lymphadenopathy [1–3]. The localized nature of the bone lesions in patients with cherubism is unexpected as the disorder is associated with heterozygous germline mutations in the gene that is widely expressed throughout the osteoimmune system [1,2,4–7]. SH3BP2 is a complex adaptor protein, with an N-terminal pleckstrin homology, a 10 amino acid Src Homology 3 (SH3) binding domain, and a C-terminal SH2 domain [8].

RANKL initiates the development and function of osteoclasts [9,10] and among the various transcriptional regulators that are activated by RANKL signaling in osteoclast precursor cells is NFATc1, a member of a small family of ubiquitous transcription factors [11–13]. NFAT phosphoproteins, extant in the cytoplasm, are activated by calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase which leads to NFAT translocation into the nucleus [14,15]. NFATc1 plays a critical role in osteoclast development. Ishida et al. first showed that NFATc1 was highly induced after sRANKL treatment of RAW 264.7 osteoclast precursors, and that inhibiting NFATc1 activity by either antisense oligonucleotides or the calcineurin inhibitor cyclosporine A suppressed osteoclast differentiation *in vitro* [16]. Similarly, Takayanagi et al. demonstrated that NFATc1 is induced in osteoclast precursors by sRANKL stimulation using DNA microarray analysis and that embryonic stem cells deficient in NFATc1 were unable to differentiate into osteoclasts *in vitro* [17]. More recently, Hirotani et al. showed that forced expression of a constitutively active form of NFATc1 is sufficient to induce RAW 264.7 osteoclast precursor cells to undergo differentiation into mature osteoclasts [18]. These results suggest that NFATc1 acts coordinately to regulate osteoclast differentiation although it is still obscure what molecules lie upstream of NFATc1 activation [19–21]. Previous studies in T cells have shown that overexpression of SH3BP2 activates transcription of a hybrid reporter gene in which luciferase expression is regulated by a promoter region containing the NFAT binding sequence from the interleukin 2 (IL-2) gene [22–24]. All SH3BP2 mutations described to date in patients with cherubism occur in exon 9 and correspond to a six amino acid region of the SH3BP2 protein [1,7,25,26]. The precise function of this region remains unclear, but recent work from our laboratory suggests that these missense mutations lead to a gain-of-function rather than a loss of activity [25]. This is consistent with prior observations that deletions of 4p16.3 in patients with Wolf-Hirschhorn syndrome, which result in loss of one copy of

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SH3BP2, do not result in a bone resorptive phenotype [27–29]. Here, we report that SH3BP2 participates in a phosphatidyl-inositol specific phospholipase C (PI-PLC), inositol phosphate-3 receptor (IP3R), and calcineurin dependent signaling cascade that activates calcineurin-induced nuclear translocation of NFATc1, leading to increased differentiation of TRAP-positive osteoclastic cells.

Materials and methods

Cell culture. HEK 293 T cells, Saos2 cells, and RAW 264.7 cells (American Type Culture Collection) and Jurkat T Ag cells (a generous gift from Professor Gerald Crabtree, Stanford University, Palo Alto, CA.) were maintained at 37 °C, 5% CO_2 in growth media: DMEM or RPMI (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Invitrogen). For osteoclastogenesis assays, cells were resuspended in fresh medium and cultured for 8 days in 96-well plates at a cell density of 1000 cells/well. Soluble RANKL (sRANKL) (Peprotech) was used at 100 ng/ml as indicated and cyclosporine A (Csa) (Calbiochem) at 0.4–2 μM, 2-APB (Calbiochem) at 75 μM, U-73122 and U73343 at 3 μM, as indicated. Csa, 2-APB, U-73122, or U73343 were added to the medium at the time of cell transfection and maintained in growth media thereafter.

Plasmids and transfection of RAW 264.7 cells. We obtained the mouse SH3BP2 cDNA in pMT3 plasmid from Professor Marcel Deckert (University of Nice, France) [22,23] and NFAT-luciferase reporter from Professor Gerald Crabtree [30]. RAW 264.7 cells at 40–70% confluence were transiently transfected with SH3BP2 cDNA, a hybrid reporter gene in which luciferase expression is under the control of a 3× minimal IL-2 promoter containing the NFAT binding site (NFAT-luc) [30], and a Renilla luciferase reporter as a control for transfection efficiency (Promega). Transfection was performed by electroporation using a Nucleofector (Amaxa). Twenty-four hours after transfection, luminescence was evaluated with a luminometer (Sirius Berthold). To create stable cell lines, RAW264.7 cells were co-transfected with plasmids encoding the NFAT-luciferase reporter gene and the neomycin resistance gene and cultured in the presence of G418 (500 μg/ml).

Immunofluorescent staining. RAW 264.7 cells were grown on cover slips in 6-well plates after Amaxa nucleofection as described above. After incubation at 37 °C for 24 h, the cells were fixed in 4% paraformaldehyde for 30 min, treated with 0.1% Triton X-100 for 5 min, and incubated in 5% BSA/PBS for 30 min. Cells were then stained with affinity-purified mouse anti-human NFATc1 (Abcam, 1:200) and affinity-purified rabbit anti-human SH3BP2 (custom made, 1:1000) for 1 h in PBS. Cells were then incubated first with 4 μg/ml Alexa 488 goat anti-mouse secondary antibody for 1 h in PBS, washed and incubated with 4 μg/ml Alexa 568 goat anti-rabbit secondary antibody for 1 h in PBS. The slides were then treated with DAPI, mounted and examined under confocal microscopy. Cells were photographed with a Leica TCS-SP spectral laser scanning confocal microscope with lasers set up to simultaneously visualize fluorescence at different wavelengths to compare subcellular localization of the mouse SH3BP2 and NFATc1 immunogens.

Determination of osteoclast differentiation. Osteoclast differentiation was assessed by measuring the cellular enzymatic activity of TRAP, a marker of terminal osteoclast differentiation. Specifically, cells were incubated in 0.1 ml of phosphatase substrate (3.7 mM *p*-nitrophenyl phosphate in 50 mM citrate buffer pH 4.8) in the presence of 10 mM sodium tartrate at 25 °C for 30 min. After incubation, 0.1 ml of NaOH was added to each well and the absorbance was read at 410 nm on a microplate reader.

Immunoblot analysis. For SH3BP2 immunoblots whole cell lysate was prepared in passive lysis buffer (Promega) and was assayed for total protein using BCA reagent (Bio-Rad). Protein samples (50 μg) were electrophoresed through 4–12% bis–tris polyacrylamide gels at 200 V for 1 h in MOPS buffer and transferred from the gel to a PVDF membrane. After an overnight incubation with 5% non fat dried milk (Carnation) at 4 °C, the membrane was incubated with a specific polyclonal antiserum to SH3BP2 (Rockland Immunochemicals) (1:2000) in 5% nonfat dried milk, or incubated with antiserum to β-actin (1:2000 in 5% nonfat dried milk) which was used as a control to evaluate loading per lane. Antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL, Amersham).

To analyze phosphorylated proteins, cells were scraped in lysis buffer (20 mM Tris–HCL, pH7.5, 20 mM *p*-nitrophenyl phosphate, 1 mM EGTA, 50 mM sodium fluoride, 50 μM sodium orthovanadate, and proteinase inhibitor cocktail) and lysed by freezing and thawing 3 times. Samples were electrophoresed on a 4–12% bis–tris gel (Invitrogen), 200 V for 40 min in MOPS buffer and transferred to PVDF membranes, at 100 V for 1 h. Membranes were then blocked in 5% skim milk for 1 h and incubated in anti-PLCγ2, phospho-specific Tyr^{1217} antibody or anti-Syk, phospho-specific $Tyr^{524-525}$ antibody (Oncogene) overnight at 4°C. Alternatively, the membrane was stripped and, incubated with PLCγ2, Syk (Cell Signaling), SH3BP2 (custom made antibody) and β-actin antibodies (Sigma) at 4°C overnight.

Statistics. Statistical evaluation of the data used the analysis of variance (ANOVA) and Fisher's protected least significant difference multiple comparison test to determine significance between groups.

Results and discussion

In previous studies we found that RAW 264.7 cells express native SH3BP2, as determined by RNA blot and protein immunoblot, at levels similar to those in Jurkat T Ag cells, HEK 293 T cells, Saos2 cells, and human giant cell tumors confirming that SH3BP2 could potentially participate in signaling pathways that induce osteoclastogenesis [31].

Since SH3BP2 is known to stimulate NFAT activity, and NFATc1 is a master regulator of osteoclastogenesis, we examined NFATc1 expression by immunoblot in response to sRANKL and then by immunofluorescence in sRANKL-stimulated RAW 264.7 cells that had been transiently transfected with SH3BP2. SH3BP2, in sRANKL-stimulated cells, did not increase the total amount of NFATc1 further but rather increased NFATc1 nuclear translocation (Fig. 1A) compared to a negative control stimulated with sRANKL but with empty vector transfected rather than SH3BP2 (Fig. 1B).

There was no consistent activation of NFAT-luc in SH3BP2 transfected RAW 264.7 cells that were cultured in the absence of sRANKL. However, when sRANKL (100 ng/ml) was added to cultured cells transfected with the wild-type SH3BP2 cDNA, NFAT-luc activity increased to 2- to 3-fold greater than in cells that had been transiently transfected with empty vector and treated with sRANKL (Fig. 1C) ($p < 0.0001$).

To determine whether over expression of SH3BP2 had an effect on osteoclastogenesis, we measured TRAP activity after RAW264.7 cells had been cultured for 1 week in the absence or presence of 100 ng/ml of sRANKL, after transfection with either an empty vector or mouse SH3BP2 (Fig. 1D). TRAP activity was significantly increased by treatment with sRANKL, and there was an even greater increase in sRANKL-induced TRAP activity in RAW264.7 cells that had been transiently transfected with SH3BP2 compared to empty vector ($p < 0.001$) (Fig. 1D). These results correlated with the synergistic effect of SH3BP2 and sRANKL on activation of the NFAT-luciferase reporter gene (Fig. 1C). However, the NFAT activation was not increased with transient transfection of NFAT-luc alone and sRANKL treatment. Moreover, previous data showed that