Tyrosine Phosphorylation Is Required for IκB Kinase-β (IKKβ) Activation and Function in Osteoclastogenesis*

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The transcription factor NF-κB is crucial for numerous cellular functions such as survival, differentiation, immunity, and inflammation. A key function of this family of transcription factors is regulation of osteoclast differentiation and function, which in turn controls skeletal homeostasis. The IκB kinase (IKK) complex, which contains IKKα, IKKβ, and IKKγ, is required for activation of NF-κB, and deletion of either IKKα or IKKβ resulted with defective osteoclast differentiation and survival. We have recently investigated the details of the mechanisms governing the role of IKKβ in osteoclastogenesis and found that constitutively active IKKβ in which serine residues 177/181 were mutated into negatively charged glutamic acids instigates spontaneous bona fide receptor activator of NF-κB ligand (RANKL)-independent osteoclastogenesis. To better understand and define the functional role of IKKβ domains capable of regulating the osteoclastogenic activity of IKK, we investigated key motifs in the activation T loop of IKKβ, which are potentially capable of modulating its osteoclastogenic activity. We discovered that dual serine (traditional serine residues 177/181) and tyrosine (188/199) phosphorylation events are crucial for IKKβ activation. Mutation of the latter tyrosine residues blunted the NF-κB activity of wild type and constitutively active IKKβ, and tyrosine 188/199-deficient IKKβ inhibited osteoclastogenesis. Thus, tyrosines 188/199 are a novel target for regulating IKKβ activity, at least in osteoclasts.

Nuclear factor κ-B (NF-κB)3 comprises a family of transcription factors crucial for numerous cellular functions including survival, differentiation, and apoptosis (1, 2). This family of transcription factors is ubiquitously expressed in various cell types of the body and regulates expression of numerous gene products including its own (3–6). Advances made in the last decade have implicated NF-κB as an effector of a wide range of inflammatory diseases, including rheumatoid arthritis and inflammatory bone erosion (5, 7, 8). NF-κB is regarded as the centerpiece of the inflammatory process and induces expression and production of pro-inflammatory and osteoclastogenic mediators (5, 9, 10). Furthermore, it has been established that persistence of NF-κB activation in inflammatory settings, which is accompanied with secretion of a wide array of inflammatory mediators, leads to dysregulation of skeletal homeostasis primarily through accelerated pathologic bone loss because of exacerbated osteoclast recruitment and activity (7–9). On the other hand, using gene deletion studies, the role of NF-κB in osteoclastogenesis was established and involves most members of this family (11).

The transcription factor NF-κB family is composed of several members including p50, p52, RelA/p65, RelB, c-Rel, the precursors NF-κB1/p105 and NF-κB2/p100 (which undergo processing into p50 and p52, respectively), and the inhibitory subunits IκBα, IκBβ, and IκBε. Activation of NF-κB dimers is regulated by the inhibitory proteins, of which IκBα has been widely investigated (4). Under unstimulated conditions, most of the NF-κB is bound to IκBα and retained in the cytosol in its inactive form. Various stimuli, such as TNF, IL-1, UV, endotoxins, lymphotoxins, viruses, the osteoclastogenic factor RANK ligand (RANKL), and other factors, prompt activation of a proximal kinase complex, leading to phosphorylation and dissociation of IκBα from the NF-κB complex, allowing nuclear translocation of the transcription factor (4, 12). Phosphorylation of IκBα occurs on N-terminal serine residues and was found to be induced by a large IκBα serine kinase (IKK) complex. The predominant IKK complex found in most cells contains two catalytic subunits, IKKα (also known as IKK1) and IKKβ (IKK2), and a regulatory subunit, IKKγ (also known as NEMO) (13–16). Although the catalytic serine kinases IKKα and IKKβ were found to target serines 32 and 36 of the IκBα (and p100 processing by IKKα), NEMO was found to act as a scaffold protein. NEMO contains several protein interaction motifs with no apparent catalytic domains, yet it is essential for staging the assembly of the IKK signalosome (17, 18).

IKKβ is the dominant activator of the classical NF-κB, mediates the effects of pro-inflammatory and pro-osteoclastic factors, and as such, plays a pivotal role in inflammatory responses. Recent studies have shown that IKKβ is crucial for osteoclastogenesis and mediates osteolysis (19, 20). In this regard, deletion of IKKβ in the myeloid lineage abrogates osteoclastogenesis in vitro and in vivo (19, 20). Consistent with this role, inhibiting IKKβ activation abolished osteoclastogenesis, inflammatory osteolysis, and inflammatory arthritis in various models of inflammation (21–23). Furthermore, dele-
tion of IKKβ renders immune cells, macrophages, and osteoclast progenitors susceptible for TNF-induced apoptosis through a gain of function in the c-Jun N-terminal kinase (JNK) pathway. This is supported by the findings that inhibition of TNF or JNK restores the osteoclastogenic and inflammatory potential of these cells (19, 20).

IKKβ is activated by proximal kinases, the identity of which has not been fully clarified. Several in vitro studies using mouse embryonic fibroblasts have implicated TAK1 as a potential activator of IKKβ (24, 25). However, the proximal events of NF-κB activation are rather complex and involve a series of polyubiquitination events culminating with activation of IKKβ (24, 25). The vast majority of published studies suggest that IKKβ undergoes serine phosphorylation on residues Ser-177/181 located in the amino-terminal activation T loop. In support of this mechanism, substitution of two of these serine residues to alanines hinders the activation of IKKβ (1). Conversely, substitution of the same serine residues to negatively charged glutamic acids renders IKKβ constitutively active. This phosphomimetic form is biologically superactive in various cellular targets (26, 27).

In a recent study, we have discovered that this phospho-mimetic form of IKKβ is capable of inducing the classical NF-κB pathway in osteoclast progenitors independent of the entire proximal signaling of RANK, TNF receptor, and IL-1 receptor. Importantly, this signaling was also found to be independent of other NF-κB partners including IKKα, NEMO, and Rel-B (27). To provide better understanding of the mechanism(s) underlying IKKβ activation, we examined the amino-terminal activation T loop of this kinase with further scrutiny. We observed that tyrosines 188 and 199, which are juxtaposed to serines 177/181, were found to be conserved among different kinases, including IKKα, AKT, cAMP-dependent protein kinase, and all protein kinase C isoforms (28, 29). Interestingly, using osteoclast progenitors, we show that IKKβ undergoes dual tyrosine and serine phosphorylation in response to RANKL and TNF. This observation is supported by an earlier study in which, using A549 cells, in vitro transfections showed that IKKβ undergoes tyrosine phosphorylation on residues 188/199 (30, 31). To better understand the role of tyrosines 188/199 in IKKβ activation mechanism and cellular function, we engineered various forms of IKKβ in which tyrosines 188/199 and serines 177/181 were substituted at various combinations. Our findings suggest that activation of IKKβ requires dual phosphorylation of tyrosines 188/199 followed by serines 177/181. Furthermore, interruption of tyrosines in the activation loop hinders the activity and function of all forms of IKKβ including the constitutively active IKKβSEYF form. Most importantly, tyrosine-mutated IKKβ inhibits expression of inflammatory mediators and arrests osteoclastogenesis.

**EXPERIMENTAL PROCEDURES**

Animals and Cells—All mice were housed in a controlled barrier facility at Washington University (St. Louis, MO). Floxed IKKβ (32) mice were from Dr. Pasparakis (University of Cologne, Germany). For in vitro experiments, wild-type C57BL/6 mice at 5–6 weeks of age were used.

Plasmids—pMx retroviral expression plasmid was from Dr. T. Kitamura (University of Tokyo, Japan). Mouse cDNA for IKKα was kindly provided by Dr. Kenneth Marcu (Stony Brook, NY). IKKβ cDNA was purchased from ATCC. All expression constructs were subcloned into pMx using standard techniques. The following mutations were generated using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Below are the primer sequences used to generate the mutants, with lowercase letters designating the site of mutation: 5′-GAG GTG CAT CAG GCC gaa CTG TGCC ACg gaa TTT gTG gGG ACT CTGC-3′, and reverse, 5′-GCA GAG TCC CCA AAA Att cCG TGC ACA GtT cGC CCT GCA GCC GCTC-3′ (note that the constitutive activating effect of this mutation of IKKβ has been established previously (33, 34)). IKKβSYFF forward, Y188_199F f, 5′-GGG ACT CTG CAA ttc CTG CAG GCC CCA CCTG TCG GAG CAG AGG TAA ACC CTG G-3′, and reverse, IKKβ_K44M_r, 5′-CAC GTT gaa CTt CTG CTC CAG AAG CTC TGG CGC CAG gaa TTG CAG AGT CCC-3′. To generate IKKβSEYF (quadruple mutant = S177/E181F/Y188F/Y199F) the IKKβSEYF backbone was used as a template with the IKKβSYFF primers. IKKβSM (IKKβ_K44M_f, 5′-GTG AAC AGA TCG CCA TCA GTC CAC GGT ACC GCT-3′) or IKKβ_K44M_r, 5′-GCT CCT GTC GCC GAC AGG AGC-3′, and IKKβ_K44M_f, 5′-GCT CCT GTC GCC GAC AGG AGC-3′.

Generation of Monocytes/Macrophages (Osteoclast Progenitors/Precursors)—Marrow was flushed from long bones into α-minimum essential medium (MEM). Cell pellets were resuspended in whole medium (α-MEM with 1% penicillin/streptomycin, 10% heat-inactivated fetal bovine serum (FBS)). Monocytes/macrophages were produced by growing cell suspensions in the presence of 10 ng/ml M-CSF. Monocytes/macrophages were allowed to proliferate for 3 days at 37 °C in 5% CO₂, at which point they were infected with retrovirus. Cells (grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 10 ng/ml M-CSF, penicillin/streptomycin, and 4 μg/ml hexadimethrine bromide). Twenty-four h after infection, cells were selected in α-MEM containing 10% FBS, 10 ng/ml M-CSF, penicillin/streptomycin, and 2 μg/ml puromycin for 72 h, at which point selection medium was removed, and cells were washed and grown for 24 additional h without puromycin. At this point, cells were lifted, counted, and plated for downstream experiments.

Generation of Retrovirus—The use of Plat-E retrovirus packaging cells stably expressing retroviral structural proteins gag-pol and env for transient production of high titer retrovirus was described previously (35). Briefly, 8 μg of pMx vectors expressing our gene of interest were transfected into 5 million Plat-E cells (grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 10 ng/ml M-CSF, and penicillin/streptomycin) using FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Twenty-four h after transfection, medium was exchanged to remove transfection reagent. Twenty-four and 48 h after medium exchange, supernatant was collected and pooled for infection of monocytes (see above).
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*In Vitro Osteoclastogenesis—* For osteoclastogenesis assays, 3 × 10^4 monocytes were plated in 200 μl of α-MEM with 10% FBS. IKKβα225CSE-expressing cells were cultured in 10 ng/ml M-CSF, whereas GFP- and IKKβWT-expressing cells were cultured in 10 ng/ml M-CSF plus 100 ng/ml RANKL for 4 days. At this point, cells were fixed and TRAP-stained using the leucocyte acid phosphatase kit (Sigma). TRAP-positive cells with three or more nuclei were scored as osteoclasts.

**RNA Isolation and cDNA Production**—RNA was isolated from macrophage or osteoclast cultures using the total RNA isolation mini kit (Agilent Technologies, Santa Clara, CA) according to the manufacturer’s instructions. Reverse transcription was described previously (19).

**Quantitative Real-time PCR**—Quantitative real-time PCR procedure was described in detail previously (19).

**Western Blotting**—The Western blot procedure was described previously (19). One million cells were used for protein extraction and demonstration of protein expression.

**Microscopy**—Cells were imaged under white or ultraviolet light on an inverted microscope (Olympus IX-51). Digital images were captured using a CCD camera (Olympus DP70, 12 megapixel resolution).

**Statistics**—A Student’s two-tailed t test for comparison between means was used for all analyses.

**RESULTS**

**IKKβ Undergoes Dual Serine and Tyrosine Phosphorylation**—Serine phosphorylation of IKKβ at residues Ser-177/181 is crucial for its activation. In fact, substitution of these two serines into alanines renders this protein insensitive to stimuli, non-phosphorylatable, and catalytically inactive. Conversely, substituting serines 177/181 into negatively charged amino acid such as glutamic or aspartic acid render IKKβ constitutively active. This form of active IKKβ phosphorylates target proteins and activates the downstream canonical NF-kB subunits in complete absence of external stimuli. Surprisingly, we have recently discovered that this constitutive activity of IKKβ empowers spontaneous osteoclastogenesis, which is independent of all known proximal signals including RANK stimulation (27). To better understand the mechanism underlying the phosphorylation events occurring in the activation loop of IKKβ that are crucial for its osteoclastogenic activity, we tested the potential contribution of other potentially important neighboring motifs. In this regard, phosphorylation of IKKβ on tyrosine residues located in the activation T loop has been suggested (31); however, neither functional significance nor meaningful mechanistic insights have been provided.

In this study, we contemplated that phosphorylation of tyrosines 188/199 located in close proximity to serines 177/181 in the activation loop of IKKβ may regulate activation of this kinase. To provide the foundation for this assumption, we first present evidence that IKKβ undergoes tyrosine phosphorylation in osteoclast progenitors treated with RANKL or TNF (Fig. 1). Tyrosine phosphorylation of IKKβ was enhanced severalfold when compared with untreated (lane 1, denoted C) condition. This tyrosine phosphorylation event was specific, as supported by the presence of tyrosine phosphorylation of the phosphatidylinositol 3-kinase subunit p85 (positive control) under TNF-treated conditions and absence of such phosphorylation with IgG (negative control). To determine the relevance and specificity of this event, we examined tyrosine and serine phosphorylation of IKKβ in the presence of the tyrosine kinase inhibitor PP2. Using antibodies specific for the T-loop serine and tyrosine residues, we demonstrate that RANKL-induced serine and tyrosine phosphorylation, as evident after 15 and 30 min of stimulation respectively, was entirely obliterated in the presence of the tyrosine kinase inhibitor PP2 (Fig. 2A), whereas protein levels of IKKβ remained unchanged. This result suggests that serine and tyrosine phos-
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Tyrosines 188/199 eliminate activity of IKKβSSEE and IKKβWT.

293 HEK cells were transfected with NF-κB luciferase promoter with the various IKK constructs or empty vector (VEC) as shown. Cells were then left unstimulated (Unstim) or treated with TNF or lipopolysaccharide (LPS) for 4 h and lysed, and luciferase activity (CLU) was measured. Error bars indicate S.E.

Meaningful activity, and baseline NF-κB transcriptional activity elicited by IKKβSSEE was not inducible, as expected. Surprisingly and consistent with our previous observations, NF-κB transcriptional activity was dramatically reduced when single or double tyrosine mutations were introduced. More importantly, tyrosine mutation of constitutively active IKKβ (IKKβSSEE) rendered this kinase entirely inactive (Fig. 3), supporting the notion that integrity of the T-loop tyrosines is obligatory for IKKβ activity. Interestingly, NF-κB activity under conditions of the absence of amino-terminal tyrosine residues was below basal activity values, suggesting that tyrosine-mutated IKKβ forms may act as dominant negative proteins. Taken together, this finding suggests that serine phosphorylation and tyrosine phosphorylation of IKKβ are coupled events.

Tyrosines 188/199 Are Essential for the Inflammatory and Osteoclastogenic Activity of IKKβ—Having established that mutation of tyrosines 188/199 abolishes NF-κB transcriptional activity, we asked whether this inhibition will impact the signaling of IKKβ as an inflammatory signal inducer. Indeed, using quantitative PCR, we demonstrate that although IKKβSSEE induces mRNA levels of TNF and IL-1β, tyrosine-mutated wild-type (WT) or SSEE forms of IKKβ fail to do so (Fig. 4, A and B).
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We have shown that IKKβ is crucial for osteoclastogenesis and that constitutively active IKKβ stimulates spontaneous osteoclastogenesis. Based on these findings, we surmised that if our hypothesis is correct, we expect that mutating tyrosines 188/199 in IKKβWT and IKKβSSEE may alter their function in osteoclastogenesis. To test this proposition, we employed the various IKKβ modified forms and tested their osteoclastogenic activity using the osteoclast formation assay. For this experiment, all relevant IKKβ forms were expressed retrovirally to facilitate efficient delivery to osteoclast progenitors. Wild-type osteoclast progenitors expressing IKKβWT generate osteoclasts in response to RANKL treatment. In contrast, when WT osteoclast progenitors were infected with tyrosine mutant IKKβ, their osteoclastogenic response was significantly impaired. Furthermore, we have shown that IKKβSSEE potently induces osteoclastogenesis regardless of RANKL (27). Even this powerful response was dramatically impaired when tyrosine mutations were introduced in the IKKβSSEE backbone (Fig. 5, A and B).

As a proof of principle, we also tested the potential of tyrosine mutated IKKβ forms to rescue osteoclastogenesis by IKKβ-null cells (without RANKL). To this end, osteoclast precursors derived from IKKβ-null mice were infected with various forms of IKKβ in which serines 177/181 or tyrosines 188/199 were mutated at different combinations. In addition, a constitutively active form of IKKα (right bottom panel) was used as a negative control. As expected, only IKKβSSEE was capable of supporting osteoclastogenesis by IKKβ-null osteoclast progenitors. However, when Tyr-188/199 were mutated (S177E/S181E/Y188F/Y199F), the osteoclastogenic activity of this constitutively active IKK form was nullified (Fig. 5, C and D). Interestingly, similar to the tyrosine mutated form, IKKβ in which serines 177/181 were mutated into alanines failed to rescue osteoclastogenesis.

Having demonstrated that PP2 inhibits tyrosine and serine phosphorylation of IKKβ (Fig. 2A), we asked whether this approach carries physiological significance in osteoclastogenesis. To address this prospect, we conducted an osteoclast formation assay in the presence of increasing doses of PP2 using both RANKL-stimulated and IKKβSSEE-induced osteoclastogenesis (Fig. 6, A and C). Although the effect of the carrier DMSO on osteoclasts was indistinguishable from control, PP2 dose dependently inhibited osteoclast formation (Fig. 6, A and B) without affecting cell survival (Fig. 6C). The effect was significant at submicromolar concentrations and maximal at 1 μM. At this optimal concentration, PP2 inhibited RANKL-stimulated and IKKβSSEE-induced osteoclastogenesis entirely (Fig. 6C). We also tested the effect of administering a tyrosine phosphorylase directly to osteoclast precursors. Specifically, cells were infected with a retroviral SHP1 or its dominant negative form SHP1(C435S) and subjected to an osteoclast formation assay. The results indicate that although SHP1 significantly inhibited osteoclast formation, its dominant negative form, which has been reported to inhibit endogenous SHP1 (36), appears to enhance osteoclastogenesis (Fig. 6C, bottom panels). These latter data suggest, but do not provide definitive proof, that the tyrosine phosphorylase SHP1 may regulate IKKβ activity. Further studies are required to investigate the details of this mechanism. Taken together, these observations suggest that tyrosine phosphorylation and serine phosphorylation of IKKβ are pivotal for its osteoclastogenic activity. In parallel experiments, we show that although IKKβSSEE (similar to RANKL) induces the osteoclast differentiation markers TRAP and NFATc1, mutation of Tyr-188/199 abolishes this expression (Fig. 7, A and B). These observations were further supported biochemically where we observed that mutating Tyr-188/199 abolished expression of the osteoclast markers NFATc1, RelB, c-Fos, and β3-integrin, which was induced by the IKKβSSEE form and by RANKL-generated osteoclasts (compare lane 7 with lanes 5 and 8, respectively) (Fig. 7C). Thus, tyrosines 188/199 are crucial for IKKβ function as osteoclastogenic mediators.

DISCUSSION

The activation loop of IKKβ contains unique elements crucial for the function of this kinase. The most studied elements are serines 177/181 and lysine 44. It has been established that serine kinases, such as TAK-1, phosphorylate IKKβ at residues 177/181. This event is essential for ATP binding by Lys-44, which is a key step in the activation of IKKβ. Mutational studies have confirmed that substitution of serines 177/181 to alanines (S177A/S181A) or lysine 44 to methionine (K44M) abrogate the activity of this kinase. Conversely, substitution of serines 177/181 to negatively charged glutamic acids (S177E/S181E) results in the constitutively active form of IKKβ because of the phospho-mimetic nature of glutamic acids (37, 38).

Several studies have demonstrated that constitutively active IKKβ is capable of executing biological functions in a ligand-free fashion at levels and durations exceeding stimulus-driven activation (26, 39). In addition, constant and continuous hyperactivity of IKKβ, typical for IKKβSSEE, often leads to dysregulated functions and may pave the way for various pathologies. In this regard, we have shown recently that when introduced into osteoclast progenitors, this active form of IKKβ leads to differentiation of bona fide osteoclasts in the complete absence of RANK signaling. This is consistent with a recent study by Sasaki et al. (26), which reported that activation of the canonical NF-κB pathway by constitutively active IKKβ renders B cell survival independent of B cell activation factor signaling cascade. Furthermore, constitutive IKKβ signaling (canonical NF-κB signaling), which is considered a hallmark of various B cell lymphomas, leads to the accumulation of resting B cells and supports their proliferation and survival upon activation. Therefore, regulation of this stimulus-independent activity of IKKβ is crucial to regulate cellular functions, specificity of which appears to be dictated by the cellular context.

In our study, we examined key elements in the activation loop of IKKβ, which has been overlooked or underappreciated, and investigated their potential contribution to IKKβ activation and regulation. In this regard, tyrosine residues 188/199 are within the activation T loop of IKKβ and are in close proximity to serines 177/181. The fact that the tyrosine kinase c-Src binds to IKKβ in endothelial cells suggests that tyrosine phosphorylation of IKKβ is potentially important for regulation of its activity. Indeed, we find that mutating these two key tyrosine residues in the activation loop renders IKKβ inactive. Even more strikingly is the finding that this double tyrosine residue mutation nullifies the activity of constitutively active IKKβ.
Tyrosine-mutated WT-IKK/β\(^{YYFF}\) and IKK/β\(^{SSEE/YYFF}\) (SEYF) inhibit osteoclast formation in wild-type cells and fail to rescue osteoclastogenesis by IKK/β-null cells. Wild-type (A) or IKK/β-null (C) osteoclast progenitors were infected with retroviral IKK/β forms as shown. Cells were then cultured in the absence or presence of RANKL for 4 days, after which cultures were fixed and TRAP-stained to visualize osteoclasts. Multinucleated (≥3 nuclei/cell) osteoclasts were counted from four wells per condition and averaged (B and D). Experiments were repeated at least three times and yielded similar results. * denotes \(p < 0.05\), and ** denotes \(p < 0.005\) when compared with WT conditions. Error bars indicate S.E.
This observation led us to posit that tyrosine phosphorylation on residues 188/199 is a crucial step that facilitate serine phosphorylation of IKK\(\beta\). Moreover, our data suggest that intact serines 177/181, or when substituted into glutamic acid (SS \(\rightarrow\) EE), are not sufficient to maintain activation of the classical NF-\(\kappa\)B pathway when tyrosines 188/199 are substituted with phenylalanines. These observations suggest that a number of scenarios are possible. First, phosphorylation of tyrosines 188/199 may serve as a first event in a multistep process leading to serine phosphorylation and ATP binding culminating with activation of the kinase. Second, phosphorylation of tyrosines 188/199 may be essential for maintaining a structural change that facilitates activation of IKK\(\beta\). Third, it is possible that phospho-tyrosines 188/199 act as a docking site for the SH2-containing protein that consequently contributes to IKK\(\beta\) activation. This possibility remains to be verified experimentally.

By way of analogy, phosphorylation of conserved tyrosine residues was found to be crucial for activation of a large number of kinases including protein kinase B/Akt, PDK1, cAMP-dependent protein kinase, and over a dozen isoforms of protein kinase C (28, 29, 40).

We have shown previously that the tyrosine kinase c-Src phosphorylates the IKK substrate I\(\kappa\)B\(\alpha\) on the amino-terminal residue Tyr-42 (41). Subsequent studies in various systems have confirmed tyrosine phosphorylation of I\(\kappa\)B\(\alpha\) and implicated c-Src family members in such process (42–45). We have confirmed the notion that c-Src or a closely related tyrosine kinase associates with the IKK complex and that IKK\(\beta\) undergoes substantial tyrosine phosphorylation on residues 188/199. The fact that both IKK\(\beta\) and its substrate I\(\kappa\)B\(\alpha\) associate with and undergo tyrosine phosphorylation, events that appear in our studies as important for osteoclastogenesis, underscores the significance of this mechanism for regulating osteolysis. However, future studies will determine whether tyrosine phosphorylation of both proteins is dependent.

We demonstrated the functional relevance of mutating tyrosines 188/199 by examining the inflammatory and osteoclastogenic activities of IKK\(\beta\). TNF and IL-1\(\beta\) are hallmarks of the inflammatory response, which are induced by canonical NF-\(\kappa\)B activation. Our data demonstrate that levels of these two cytokines is blunted in osteoclast progenitors expressing IKK\(\beta^{YYFF}\) or IKK\(\beta^{SEYF}\), bolstering the notion that intact tyrosines 188/199 are essential for this activity by IKK\(\beta\).

More importantly, our data confirm that IKK\(\beta^{YYFF}\) acts as a dominant negative protein that hinders osteoclastogenesis induced by RANKL or by IKK\(\beta^{SEYF}\), as shown by osteoclast differentiation assay and by regulating mRNA and protein expression of key osteoclast differentiation markers. Consistent with these findings, administration of the tyrosine kinase inhibitor PP2 or expression of the tyrosine phosphatase SHP1, both

![Figure 6](image.png)

**Figure 6. The tyrosine kinase inhibitor PP2 and tyrosine phosphatase SHP1 inhibit osteoclastogenesis.** Osteoclast precursors were treated with RANKL or infected with IKK\(\beta^{SEYF}\) and cultured at the presence of PP2 (at the concentrations shown) or the carrier DMSO. A, cultures were fixed on the 4th day of culture and stained for TRAP expression. B, the osteoclasts in panel A were counted under a light microscope. Each bar represents the average of four wells. The experiment was repeated three times with consistent results. * denotes \(p < 0.05\), and ** denotes \(p < 0.001\). Error bars indicate S.E. C, upper panels represent osteoclasts from RANKL-treated cells in the absence or presence of PP2. Middle panel represents cells infected with retroviral IKK\(\beta^{SEYF}\) and cultured in the absence or presence of PP2 as indicated. Lower panels represent cells infected with retroviral SHP1 or mutant SHP1 (m-SHP1) and subjected to osteoclastogenic conditions in the presence of RANKL. Arrows mark multinucleated osteoclasts.
with previous findings by our group and others demonstrating that IKKβ is crucial for osteoclast differentiation and function and plays a key role in the regulation of skeletal integrity.

Taken together, our data propose a potentially novel mechanism for IKKβ activation. This mechanism requires intact tyrosine residues at positions 188/199 in the activation loop, phosphorylation of which facilitates and/or acts in concert with phosphorylation of serines 177/181 for proper activation of IKKβ. Furthermore, tyrosines 188/199 appear to be crucial for this process because their mutation blunts the activity of the serine phospho-mimetic form of IKKβ (IKKβSEYF). Finally, this mechanism provides another level at which activation of IKKβ can be regulated and presents a potential target for therapeutic intervention.

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FIGURE 7. Tyrosine-mutated WT-IKKβ (IKKβYYFF) and IKKβSEYFF (SEYF) inhibit osteoclast differentiation markers. Osteoclast progenitors were infected with IKKβ retroviral forms as indicated for 6 days. Cells were then lysed, and mRNA expression and protein expression of osteoclast differentiation markers TRAP, NFATc1, β3-integrin, RelB, and c-Fos were measured by quantitative PCR (A and B) or Western blot (C). Total lysate from RANKL-induced osteoclasts was used as a reference (panel C, last lane, marked with OC+). Error bars indicate S.E. Unstim, unstimulated. RL denotes RANKL.
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