TAT Fusion Proteins Containing Tyrosine 42-deleted IkBα Arrest Osteoclastogenesis

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In most circumstances, NF-κB, which is essential for osteoclastogenesis, is activated following serine 32/36 phosphorylation of its cytosolic inhibitory protein, IkBα. In contrast to other cell types, IkBα, in bone marrow macrophages (BMMs), which are osteoclast precursors, is tyrosine-phosphorylated by c-Src kinase. To address the role of IkBα phosphorylation in osteoclastogenesis, we generated TAT fusion proteins containing wild-type IkBα (TAT-WT-IkBα), IkBα lacking its NH2-terminal 45 amino acids (TAT-IkBαΔ46–317), and IkBα in which tyrosine residue 42, the c-Src target, is mutated into phenylalanine (TAT-IkBα(Y42F)). TAT-IkBα efficiently enters BMMs, and the NF-κB-inhibitory protein, once intracellular, is functional. While TAT-WT-IkBα only slightly inhibits osteoclastogenesis, osteoclast recruitment is diminished >80% by TAT-IkBα(Y42F), an event mirrored by dentin resorption. The fact that TAT alone does not impact osteoclastogenesis, which also resumes following withdrawal of TAT-IkBα(Y42F) establishes that the mutant’s anti-osteoclastogenic properties do not reflect toxicity. Affirming a functional role for IkBα(Tyr42) in osteoclastogenesis, TAT-IkBα(Y42F) is as efficient as TAT-IkBα(Y42F) in blocking osteoclast differentiation. Thus, dominant-negative IkBα constructs block osteoclastogenesis, and Tyr42 is essential to the process, increasing the possibility that nonphosphorylatable forms of IkBα may be a means of preventing pathological bone loss.

Osteoclastogenesis is governed by mediators of the inflammatory response, such as the cytokines, tumor necrosis factor-α (TNF)§ and interleukin-1 (1–3). Moreover, the bacterial product, lipopolysaccharide, in murine marrow culture, induces expression of TNF, which, via its p55 receptor, activates c-Src, in bone marrow macrophages (BMMs) and prompts their differentiation into mature osteoclasts (4). These inflammatory cytokine-mediated events typically involve activation of the transcription factor NF-κB. The fact that deletion of the p50 and p52 subunits of NF-κB leads to osteoporosis, due to failed osteoclastogenesis, establishes that activation of this transcription complex is essential for commitment of BMMs to the osteoclast phenotype (5).

The NF-κB family is composed of five members that can homo- or heterodimerize following activation (6). The transcription factor resides in its inactive form in the cytoplasm avidly bound to the inhibitory protein IkBα. In a variety of cell types, extracellular signals lead to phosphorylation and degradation of IkBα, thereby releasing NF-κB. The transcription factor then translocates to the nucleus, binds to DNA sequences, and activates target genes (7). While in most circumstances IkBα is serine-phosphorylated, it can undergo phosphorylation on tyrosine 42 (8–10). This event, in TNF-induced osteoclast precursors, also leads to NF-κB release and nuclear translocation (10).

While NF-κB is clearly essential for osteoclast formation, the means by which it stimulates the process is unclear. The goals of the present exercise were, therefore, 2-fold. In the first instance, we wished to determine whether tyrosine phosphorylation of IkBα is essential for BMM differentiation into osteoclasts. Second, we asked if osteoclastogenesis, and attendant bone resorption, can be inhibited by dominant-negative IkBα, specifically that in which Tyr42 is unavailable for phosphorylation.

The planned exercise required expression of putative dominant-negative forms of IkBα in osteoclast precursors. This strategy was hampered, however, by the fact that osteoclasts, and their monocyte/macrophage progenitors, are presently impossible to efficiently transfect by traditional methods. To overcome this obstacle we took advantage of TAT, a peptide derived from the human immunodeficiency virus, type-1 transduction domain (11, 12). Proteins fused to this 11-amino acid sequence, from the human immunodeficiency virus, type-1 transduction domain, readily enters osteoclast precursors in bona fide osteoclast precursors.

Our data show that TAT-fused IkBα readily enters osteoclast precursors in a dose-dependent manner and elevates NF-κB levels in the cytosol. More importantly, IkBα lacking all phosphorylation sites, inhibits osteoclastogenesis in a nontoxic reversible manner and blocks bone resorption.

MATERIALS AND METHODS

Reagents—Polyclonal anti-IkBα and anti-NF-κB antibodies were purchased from Santa Cruz (Santa Cruz, CA). T25(OH)2D3 was provided by Dr. Milan Uskokovic (Hoffman La-Roche, Nutley, NJ). The ECL kit was obtained from Pierce. Wild-type and tyrosine 42-mutated IkBα cDNAs were a gift of Dr. Jean-Francois Peyron (Nice, France). IkBαΔ46–317 super-repressor mutant was generated by NH2-terminal deletion of residues 1–45 using a standard polymerase chain reaction approach. All other chemicals were obtained from Sigma.
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**Animals—**C57/HetN males were purchased from Harlan Industries (Indianapolis, IN).

**Cell Culture—**BMMs were isolated as described previously (4). Briefly, whole bone marrow of 4–6 week mice was collected and incubated in tissue culture plates, at 37°C in 5% CO₂, in the presence of 10 ng/ml macrophage-colony-stimulating factor (4). After 24 h, in culture, the nonadherent cells were collected and layered on a Fibro-Hypaque gradient. Cells at the gradient interface were collected and plated in α-minimal essential medium, supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in 5% CO₂, in the presence of 10 ng/ml macrophage-colony-stimulating factor, and plated according to each experimental condition.

**Osteoclast Generation—**Whole marrow cultures were plated in 48 multi-well plates at 2 × 10⁶ cells/ml/well in the presence of 10 ng/ml α-MCSF, 10 ng/ml IL-4, and 10 ng/ml 1,25-(OH)₂D₃. Cultures were supplemented with 10 ng/ml 1,25-(OH)₂D₃, and fresh media on day 4 of culture. Osteoclasts develop on days 7–8 of culture at a point where cells are fixed and TRAP-stained.

**Immunostaining—**BMMs were plated on multi-well coverslips in the absence or presence (1 h) of TAT-IκB. Cells were then fixed and stained with anti-HA antibody and detected with fluorescein secondary antibody.

**Immunoblotting—**Total cell lysates were boiled in the presence of 2× SDS-sample buffer (0.5 M Tris-HCl (pH 6.8), 10% (w/v) SDS, 10% glycerol, 0.05% (w/v) bromphenol blue, distilled water) for 5 min and subjected to electrophoresis on 8–12% SDS-polyacrylamide gel electrophoresis (18). Proteins were transferred to nitrocellulose membranes using a semi-dry blotter (Bio-Rad) and incubated in blocking solution (10% skim milk prepared in PBS containing 0.05% Tween 20), to reduce nonspecific binding. Membranes were washed with PBS/Tween buffer and exposed to primary antibodies (1 h at room temperature), washed again four times, and incubated with the respective secondary horseradish peroxidase-conjugated antibodies (1 h at room temperature). Membranes were washed extensively (5 × 15 min), and an ECL detection assay was performed following the manufacturer’s directions.

**pTAT Construct and Protein Coupling—**Various IκB constructs were cloned into the pTAT-HA bacterial expression vector described previously by Nagahara et al. (13), which contains a six-histidine tag, for easy purification, an HA tag for detection followed by the TAT transduction domain, and finally the IκB sequence. The resultant plasmid, pTAT-IκB, was transformed into the DH5α strain of *Escherichia coli*. The transformants were screened initially by restriction enzyme mapping. A recombinant containing the correct restriction fragments was then sequenced on both strands. This plasmid was then used to map the IκB gene. A recombinant containing the correct restriction fragments was then sequenced on both strands. This plasmid was then used to map the IκB gene.

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**TAT-IκB Enters Osteoclast Progenitors and Retains NF-kB—**To test TAT-IκB transduction, we treated BMMs, for 1 h, with a TAT-IκB fusion protein containing HA tag (HA-TAT-IκB), transfected by incubation, using anti-HA monoclonal antibody. HA-TAT-IκB transduced the vast majority of BMMs in culture (Fig. 1). To determine whether the HA-TAT-IκB enters BMMs in a concentration-dependent manner, we incubated osteoclast precursors with increasing amounts of the purified fusion protein. Lysates of 1-h-treated cells were immunoblotted with anti-HA monoclonal antibody. As shown in Fig. 2A, HA-TAT-IκB enters BMMs, dose-dependently.

Because IκBα normally binds to, and retains, NF-κB in the cytoplasm, we asked if TAT-IκB affects cellular localization of NF-κB. Attesting to the TAT fusion protein’s functionality, the transduced inhibitory protein, dose-dependently, retains NF-κB in the cytosol as assessed by measuring cytosolic levels of p65 NF-κB (Fig. 2B).

**TAT-IκB Proteins Block Osteoclastogenesis—**Given that NF-κB deletion blunts osteoclastogenesis, we asked whether inhibition of NF-κB activation would also block osteoclast development. We reasoned that introduction of IκBα protein, the NF-κB inhibitory protein, devoid of its functional phosphorylation sites, into osteoclast precursors might block osteoclast recruitment. To address the role of IκBα phosphorylation in osteoclastogenesis, we generated TAT fusion proteins containing wild-type IκBα (TAT-WT-IκB) and IκBα lacking its NH₂-terminal 45 amino acids (TAT-IκBΔN₃¹⁷). Marrow, placed in osteoclastogenic conditions (4), was treated, on day 3 of culture, with the various TAT-IκB constructs or with TAT alone. In some cultures the inhibitory proteins were withdrawn following 48 h of exposure. Tartrate-resistant acid phosphatase (TRAP) expression by these cultures indicates that TAT alone fails to impact osteoclast recruitment, indicating that the human immunodeficiency virus-derived peptide is nontoxic in these circumstances (Fig. 3A, row D). Osteoclast recruitment is, however, diminished >80% by TAT-IκBΔN₃¹⁷ (Fig. 3A, wells C1–C2, and B), suggesting that the functional phosphorylation sites are central to the process. The resumed osteoclast differentiation following withdrawal of the inhibitory construct establishes that the TAT fusion protein’s anti-osteoclastogenic properties are reversible (Fig. 3A, rows 3–4). In contrast to TAT-IκBΔN₃¹⁷, TAT-WT-IκB only slightly inhibits osteoclastogenesis (Fig. 3A, wells B1–B2). As expected, inhibition of osteoclast formation by TAT-IκBΔN₃¹⁷ is mirrored by arrested dentin resorption (Fig. 4). The number of resorption lacunae counted in 1 cm² was 187 ± 39 in PBS + TAT compared with 20 ± 14 in TAT-IκB-treated conditions. Furthermore, lacunae in TAT-treated conditions were well defined, wide and deep tracks.
compared with shallow focal pits in TAT-IxB-treated conditions.

Tyrosine 42-mutated IκB Inhibits NF-κB Activation and Osteoclastogenesis—Having established that deletion of the functional phosphorylation sites to IκBα blunts osteoclastogenesis, we asked if mutation of tyrosine 42, which is specifically phosphorylated in osteoclast precursors, mirrors this event. To this end we generated TAT-IκB(Y42F) and examined its cellular transduction activity. First, HA-TAT-IκB(Y42F) was added to osteoclast precursors, and the presence of the protein was examined with time by immunoblots. We find that the protein for ubiquitination and subsequent degradation. NF-κB

for different time points. The data depicted in Fig. 7A indicate that, while as expected, TNF reduces cytosolic levels of NF-κB (lane 2), tyrosine-mutated TAT-IκB prevents this reduction within 1 h, and levels of NF-κB in the cytosol are sustained up to 16 h. Reduced levels of NF-κB in days 2 and 3 (lanes 6 and 7) most likely reflect decay of the TNF signal and/or degradation of the TAT protein. The observations in Fig. 7A are further supported by our findings that TNF enhances nuclear levels of NF-κB (Fig. 7B, lane 2) and that the various IκBα proteins block it (lanes 3–5). Finally, we examined whether, similar to TAT-IκB(Y42F), addition of TAT-IκB(Y42F) to osteoclastogenic cultures blocks osteoclastogenesis. Establishing a functional role for IκBα(Y42F) in osteoclastogenesis, we find that TAT-IκB(Y42F) is as efficient as TAT-IκB(Y42F) in blocking osteoclast differentiation (Figs. 8 and 9).

FIG. 5. TAT-IκB(Y42F) remains in cells for up to 2 days. Cells were incubated with 100 nM TAT-IκB(Y42F) for the time points indicated. Cells were then lysed and immunoblotted with anti-HA antibody. In other experiments (not shown), lysates were immunoprecipitated with anti-HA antibody and blotted with anti-IκB antibody. Results of both experiments were identical.

FIG. 6. TAT-IκB(Y42F) enters cells and retains cytosolic NF-κB in a dose-dependent manner. Cells were cultured as indicated in the legend to Fig. 5 and treated with increasing doses of TAT-IκB(Y42F) as shown. Levels of transduced TAT-IκB (A) and cytosolic NF-κB (B) were measured by immunoblots using anti-HA and anti-NF-κB antibodies, respectively.

FIG. 7. Tyrosine 42-mutated IκB prevents TNF-induced nuclear translocation. A, immunoblot of nuclear (lanes 1 and 5) and cytosolic NF-κB (lanes 2 and 6) from cultures treated with increasing doses of TAT-IκB(Y42F) for the time points indicated. B, immunoblot of nuclear (lanes 1 and 5) and cytosolic NF-κB (lanes 2 and 6) from cultures treated with increasing doses of TAT-IκB(Y42F) for the time points indicated.

FIG. 8. TAT-IκB(Y42F) inhibits matrix resorption. BMNs were plated on dentin slices in 24 multi-well plates. TAT, alone, or TAT-IκB(Y42F) inhibits matrix resorption and formation. Three days later dentin slices were washed with PBS, fixed, and resorption pits visualized by electron microscopy scanning. Arrows indicate bone pits.

FIG. 9. TAT-IκB(Y42F) inhibits osteoclast formation. Three days later dentin slices were washed with PBS, fixed, and stained for TRAP expression. The intensity of the stain represents the number of TRAP-positive osteoclasts.

FIG. 10. TAT-IκB(Y42F) inhibits osteoclastogenesis. BMNs were plated in osteoclastogenic conditions. A, on day 4 of culture, cells were maintained in the absence (wells A1–A4) or presence (100 nM) of WT-IκB (wells B1–B4), TAT-IκB(Y42F) (wells C1–C4), or TAT alone (wells D1–D4). After 24 h, cells were washed and incubated with fresh media for 3 days in the absence (rows 3 and 4) or presence (rows 1 and 2) of the various peptides in their respective wells. Cultures were then fixed and stained for TRAP expression. The intensity of the red/purple stain represents the number of TRAP-positive osteoclasts.

FIG. 11. TAT-IκB(Y42F) inhibits osteoclastogenesis. BMNs were plated on dentin slices in 24 multi-well plates. TAT, alone, or TAT-IκB(Y42F) inhibits matrix resorption and formation. Three days later dentin slices were washed with PBS, fixed, and stained for TRAP expression. The intensity of the stain represents the number of TRAP-positive osteoclasts.

FIG. 12. TAT-IκB(Y42F) inhibits osteoclastogenesis. BMNs were plated on dentin slices in 24 multi-well plates. TAT, alone, or TAT-IκB(Y42F) inhibits matrix resorption and formation. Three days later dentin slices were washed with PBS, fixed, and stained for TRAP expression. The intensity of the stain represents the number of TRAP-positive osteoclasts.

FIG. 13. TAT-IκB(Y42F) inhibits osteoclastogenesis. BMNs were plated on dentin slices in 24 multi-well plates. TAT, alone, or TAT-IκB(Y42F) inhibits matrix resorption and formation. Three days later dentin slices were washed with PBS, fixed, and stained for TRAP expression. The intensity of the stain represents the number of TRAP-positive osteoclasts.
A

B

Fig. 7. TAT-IκB(Y42F) blocks TNF-induced NF-κB nuclear translocation. Cells were left untreated or exposed to TNF (10 ng/ml) in the presence or absence of TAT-IκB(Y42F) for the time points indicated (A) or with the various TAT-IκB proteins for 1 h (B). Cytosolic and nuclear fractions of the cells were prepared, and the levels of cytosolic (A) and nuclear (B) NF-κB were determined by immunoblots.

Fig. 8. Exogenously added mutated-IκB(Y42F) to marrow cultures inhibits osteoclastogenesis. Cells were plated and treated as described for Fig. 3, and a photomicrograph of TRAP-stained TAT and TAT-IκB(Y42F)-treated osteoclast cultures were recorded. Control wells treated with PBS+tAT contained 166 ± 36 osteoclasts/well compared with 41 ± 17 in TAT-IκB(Y42F)-treated wells.

Fig. 9. Nonphosphorylatable IκB proteins block osteoclastogenesis. Marrow was placed in osteoclastogenic conditions. On day 5, TAT peptide, alone, or TAT-WT-IκB, TAT-IκB(Y42F), or TAT-IκB(Y42F) (100 nM) were added for an additional 3 days. Cultures were stained for TRAP activity and the number of osteoclasts/well counted. Data are presented as the average number of multi-nucleated (≥3 nuclei), TRAP-positive cells in triplicate wells from two independent experiments. * represents p < 0.001 relative to TAT.

in turn, translocates to the nucleus to function as a transcriptional complex. On the other hand, NF-κB activation, in a restricted population, is attendant upon tyrosine IκBα phosphorylation, a process mediated by Src family kinases. For example, deletion of c-Src in osteoclast precursors or p56

T-lymphocytes inhibits NF-κB activation and interleukin-6 secretion (8, 10). Moreover, we and others (9, 10, 21) find that IκB(Y42F) is a functional phosphorylation site. In this regard, our previous studies unveiled that the tyrosine kinase c-Src, also essential for osteoclastogenesis, mediates tyrosine phosphorylation of IκBα on tyrosine residue 42, in osteoclast progenitors. This phosphorylation is followed by activation of the NF-κB transcriptional complex.

Extending these observations to a possible therapeutic approach, we reasoned that nonphosphorylatable IκBα might serve as a dominant-negative inhibitor of osteoclastogenesis. Specifically, we speculated that mutating IκBα amino-terminal phosphorylation sites might preserve the IκBα-NF-κB complex in osteoclast precursors. However, significant effect requires introduction of the mutated proteins in the vast majority of primary osteoclast progenitors, an unattainable goal by traditional techniques. To resolve this contingent, we utilized the TAT-mediated protein transduction method, developed and successfully used by our group to introduce a large number of proteins into a wide spectrum of cells, including primary osteoclast precursors (17, 22).

Our data show that dominant-negative forms of IκBα, delivered into osteoclast precursors, as TAT fusion proteins, prevent the cells’ commitment to the osteoclast phenotype. Importantly, delivery of the mutated TAT-IκB proteins is a rapid event, which is sustained for at least 24 h. Moreover, transduced IκBα mutant proteins in osteoclast precursors, dose-dependently, retain NF-κB in the cytoplasm under basal and TNF-stimulating conditions. Thus, TAT-IκB mutants appear to act as dominant-negative proteins by displacing endogenous IκBα; the later remains susceptible for phosphorylation and processing.

The biological significance of TAT-IκB delivery into osteoclast precursors is manifested by its potent anti-osteoclastogenic effect. In this regard, we document that while TAT-WT-IκB, is only slightly inhibitory, serine/serine/tyrosine-deleted and tyrosine-mutated IκBα proteins are highly effective in blocking osteoclast development. These effects correlate well with their observed effect on NF-κB inactivation. While yet to be proven, our observations suggest that the raised levels of WT-IκB, delivered as a TAT construct, are subject to phosphorylation and removal and, thus, are insufficient to block prolonged activation of NF-κB in osteoclast precursors, and hence, osteoclastogenesis continues. Additionally, the lack of impact of TAT peptide or TAT-WT-IκB on osteoclast recruitment together with reversibility of the effect underscores the nontoxic nature of these fusion proteins.

The capacity of these constructs to effectively transduce osteoclasts reinforces the potential of TAT fusion proteins as therapeutic moieties. As regard to the present constructs, preventing phosphorylation of IκBα at its NH₂-terminal domain, in general, and tyrosine phosphorylation on residue 42, in particular, substantially diminishes osteoclastogenesis. Taken with the restricted cell specificity of IκBα tyrosine phosphorylation, these observations raise the possibility that nonphosphorylatable TAT-IκB may be a means of averting pathological bone loss.

REFERENCES
1. Teitelbaum, S. L., Tondravi, M. M., and Ross, F. P. (1997) J. Leukoc. Biol. 61, 381–388
2. Teitelbaum, S. L., Tondravi, M. M., and Ross, F. P. (1996) in Osteoporosis (Marcus, R., Feldman, D., and Kelsey, J., eds) pp. 61–94, Academic Press, San Diego, CA
3. Mundy, G. R. (1996) Osteoporosis 11, 302–310
4. Abu-Amer, Y., Ross, F. P., Edwards, J., and Teitelbaum, S. L. (1997) J. Clin. Invest. 100, 1557–1565
5. Iotsova, V., Caamano, J., Loy, J., Young, Y., Lewin, A., and Bravo, R. (1997) Nat. Med. 3, 1265–1269
6. Baldwin, A. S., Jr. (1996) Annu. Rev. Immunol. 14, 649–683
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7. Baeuerle, P. A., and Henkel, T. (1994) Annu. Rev. Immunol. 12, 141–179
8. Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B., Muller-Dieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P. A., and Peyron, J. F. (1996) Cell 86, 787–798
9. Singh, S., Darnay, B. G., and Aggarwal, B. B. (1996) J. Biol. Chem. 271, 31049–31054
10. Abu-Amer, Y., Ross, F. P., McHugh, K. P., Livolsi, A., Peyron, J. F., and Teitelbaum, S. L. (1998) J. Biol. Chem. 273, 29417–29423
11. Fawell, S., Seery, J., Daikh, Y., Moore, C., Chen, L. L., Pepinsky, B., and Barsoum, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 664–668
12. Vives, E., Brodin, P., and Lebleu, B. (1997) J. Biol. Chem. 272, 16010–16017
13. Nagahara, H., Vocero-Akbani, A. M., Snyder, E. L., Ho, A., Latham, D. G., Lissy, N. A., Becker-Hapak, M., Ezhevsky, S. A., and Dowdy, S. F. (1998) Nat. Med. 4, 1449–1452
14. Vocero-Akbani, A. M., Heyden, N. V., Lissy, N. A., Ratner, L., and Dowdy, S. F. (1999) Nat. Med. 5, 29–33
15. Schwarze, S. R., Ho, A., Vocero-Akbani, A., and Dowdy, S. F. (1999) Science 285, 1569–1572
16. Lissy, N. A., van Dyk, L. F., Becker-Hapak, M., Vocero-Akbani, A., Mendler, J., and Dowdy, S. F. (1998) Immunity 8, 57–65
17. Schwarze, S. R., Hruska, K. A., and Dowdy, S. F. (2000) Trends Cell Biol. 10, 290–295
18. Laemmli, U. K. (1970) Nature 227, 680–685
19. Teitelbaum S. L. (2000) Science 289, 1504–1508
20. Franzoso, G., Carlson, L. B., Xing, L., Poljak, L., Shores, E., Brown, K., Leonardi, A., Tran, T., Boyce, B., and Siebenlist, U. (1997) Genes Dev. 11, 3482–3496
21. Mukhopadhyay, A., Manna, S., and Aggarwal, B. (2000) J. Biol. Chem. 275, 8549–8555
22. Abu-Amer, Y. (2001) J. Clin. Invest. 107, 1375–1385
