Mod Rheumatol (2004) 14:1–5
DOI 10.1007/s10165-003-0257-2

REVIEW ARTICLE

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Osteoclastic bone resorption through receptor tyrosine kinase and extracellular signal-regulated kinase signaling in mature osteoclasts

Abstract It has recently been suggested that signaling through receptor tyrosine kinases (RTKs) expressed on mature osteoclasts is involved in osteoclastic bone resorption. This study investigated the role and mechanism of two major RTKs expressed on mature osteoclasts, fibroblast growth factor receptor type 1 (FGFR1) and Tyro 3. Among the FGF receptors (FGFR1–4), only FGFR1 was detected on isolated mouse osteoclasts, while all FGFRs were identified on mouse osteoblasts. Tyro 3 was seen only in mature osteoclasts among bone cells. FGF-2 moderately stimulated pit formation by isolated rabbit osteoclasts at low concentrations ($\geq 10^{-12}$M), whereas at high concentrations ($\geq 10^{-9}$M) it strongly stimulated pit formation by unfractionated bone cells. Gas6, the ligand of Tyro 3, was expressed ubiquitously in bone cells and stimulated osteoclast function to form resorbed pits on a dentine slice. Both FGF-2 and Gas6 upregulated the phosphorylation of cellular proteins, including extracellular signal-regulated kinase (ERK), and increased the kinase activity of immunoprecipitated FGFR1 and Tyro 3, respectively, in mouse osteoclasts. The stimulation of these cytokines on mouse and rabbit osteoclast functions was abrogated by PD98059, a specific inhibitor of ERK. These results strongly suggest that these cytokines act directly on mature osteoclasts through the activation of RTKs and ERK, causing the stimulation of bone resorption.

Key words Bone resorption · Fibroblast growth factor · Gas6 · Osteoclast · Tyro 3

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Introduction

Osteoclastic bone resorption is regulated by the differentiation, function, and survival of osteoclasts. The tumor-necrosis-factor-family molecule receptor activator of the NF-κB ligand (RANKL) was identified as the membrane-associated molecule regulating osteoclast differentiation,[1–3] and the molecule involved in the differentiation step has been thought to be the main therapeutic target of osteoclastic bone resorption in rheumatoid arthritis. However, the signaling pathways regulating mature osteoclast function and survival are still controversial. A recent study using random sequence analysis of polymerase chain reaction (PCR)-amplified cDNA clones identified 14 distinct kinase-related genes in purified rabbit mature osteoclasts, and eight of these were identified as receptor tyrosine kinases (RTKs).[4] RTKs expressed on mature osteoclasts include fibroblast growth factor receptor type 1 (FGFR1), c-Fms, and Tyro 3, whose ligands, FGF-2, macrophage-colony stimulating factor (M-CSF), and the growth arrest-specific gene 6 (Gas6), respectively, are known to regulate osteoclast differentiation, function, or survival. We recently reported that FGF-2 stimulates mature osteoclast function directly through the activation of FGFR1 on mature osteoclasts.[5,6] M-CSF is also reported to stimulate the survival and chemotactic behavior of osteoclasts through the activation of its receptor, c-Fms, on osteoclasts.[7–10] Another RTK, Tyro 3, was the RTK most frequently cloned in isolated rabbit osteoclasts in the random sequence study cited above.[4] The ligand for Tyro 3 is known to be Gas6, which directly stimulates mature osteoclast function.[11] This article describes the role and mechanism of these two major RTK signals on osteoclast activation.

FGF-2/FGFR1 signaling in osteoclasts

After comparing the effects of fractionated osteoclasts and unfractionated bone cells from rabbit long bones, we re-

ported that FGF-2 at low concentrations ($\geq 10^{-12}$ M) moderately stimulated bone resorption through its direct action on osteoclasts, while at high concentrations ($\geq 10^{-9}$ M) it strongly stimulated bone resorption through its indirect action mediated by cyclooxygenase-2 induction in osteoblastic cells.[5] When mRNA and protein levels of FGFRs on osteoclasts were studied and compared with those on osteoblasts from neonatal mouse calvariae, only FGFR1 was detected on osteoclasts, while all FGFR1–4 were identified on osteoblasts in both mRNA and protein levels. This difference in the distribution of FGFRs between osteoclasts and osteoblasts might explain the difference in affinities and concentrations of the FGF-2 affecting these cells.

Figure 1 shows the time course of the effects of FGF-2 on tyrosine phosphorylation of cellular proteins in isolated mouse osteoclasts. Several proteins were selectively phosphorylated by FGF-2 (10^{-12} M) as early as 2 min. The c-Src signal in each lane indicates quantitative internal control. Western blot analyses using antibodies against specific proteins related to MAP kinase revealed that phosphorylation of extracellular signal-regulated kinase (ERK) was induced at 5 min, reached a maximum at 10 min, and was maintained for more than 30 min (Fig. 1A). Phosphorylations of p38 and JNK MAP kinases were slightly induced at 10 min only. To investigate the autophosphorylation of FGFR1 by FGF-2, the kinase activity of immunoprecipitated FGFR1 was examined by in vitro kinase assay. FGF-2 induced the kinase activity of FGFR1 at 1 min, which reached maximum at 2 min, and decreased considerably after 10 min (Fig. 1B). To examine the functional relevance of the activation

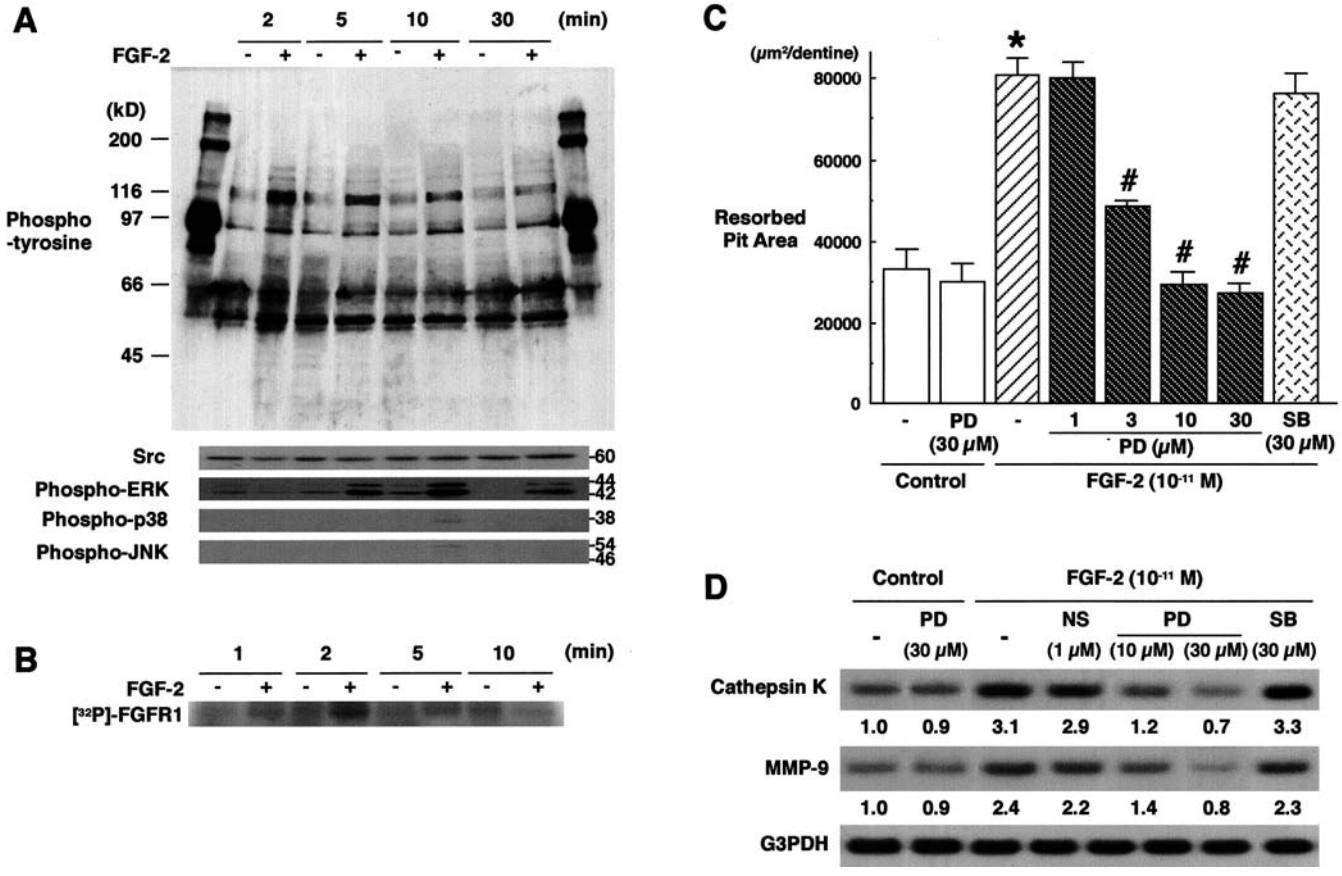

Fig. 1. Intracellular signaling through FGF-2/FGFR1 in isolated osteoclasts. **A** Western blotting of phosphotyrosine proteins and MAP kinase-related proteins in isolated mouse osteoclasts. Mouse osteoclasts formed in the co-culture were cultured with and without FGF-2 (10^{-12} M) for various periods (2–30 min) and lysed with TNE buffer. Ten μg cell lysates was subjected to 8% SDS–PAGE, and immunoblotted with polyclonal antibodies against phosphotyrosine, $p60^{v\text{-}src}$, phospho-ERK, phospho-p38 MAP kinase, and phospho-JNK, as described in Experimental procedures. **B** Tyrosine kinase activity of immunoprecipitated FGFR1 in isolated mouse osteoclasts. Isolated mouse osteoclasts were cultured with and without FGF-2 (10^{-12} M) for various periods (1–10 min), lysed with TNE buffer, and 100 μg cell lysates was immunoprecipitated with polyclonal anti-FGFR1 antibody. The samples were incubated in kinase buffer with [g-^{32}P]-ATP, and subjected to SDS–PAGE. **C** Effects of PD98059 (PD) and SB203580 (SB) on resorbed pit formation by isolated rabbit osteoclasts in the presence or absence of FGF-2. Purified mature rabbit osteoclasts were prepared and seeded onto a dentine slice. FGF-2 (10^{-11} M), PD98059 (1, 3, 10, and 30 μM), and SB203580 (30 μM) were added to the culture 1 h after seeding. After 24 h culture, the total area of pits was measured. Data are expressed as means (bars) ± SEMs (error bars) for eight cultures/group. *$P < 0.01$, significant stimulation by FGF-2; #$P < 0.01$, significant inhibition by PD98059. **D** Effects of PD98059 (PD), SB203580 (SB), and NS-398 (NS) on mRNA levels of cathepsin K and MMP-9 in the presence or absence of FGF-2 in isolated mouse osteoclasts. After incubation for 2 h, mouse osteoclasts were cultured in the presence or absence of FGF-2 (10^{-11} M), PD98059 (10 and 30 μM), SB203580 (30 μM), and NS-398 (1 μM) for 3 h. Steady-state mRNA levels were examined by Northern blot analysis. The number under each band is the treated/control ratio of the intensity of each band normalized to that of G3PDH measured by densitometry. *ERK*, extracellular signal-regulated kinase

of ERK and p38 MAP kinases by FGF-2 in osteoclasts, PD98059, a specific inhibitor of upstream kinase of ERK,[12,13] and SB203580, a specific inhibitor of p38 MAP kinase,[14,15] were added to the cultures of rabbit and mouse osteoclasts. PD98059 dose-dependently inhibited the stimulation of FGF-2 on pit formation resorbed by isolated rabbit osteoclasts to the levels of the control culture, while SB203580 (30 μM) did not affect the FGF-2 stimulation (Fig. 1C). PD98059 also inhibited the FGF-2 stimulation on cathepsin K and MMP-9 mRNA levels in isolated mouse osteoclasts, but this inhibition was not seen with SB203580 or NS-398 (Fig. 1D). Taken together, the direct action of FGF-2 in mature osteoclasts was mediated by the autophosphorylation of FGFR1, the only subtype of FGFRs expressed on osteoclasts, and the subsequent phosphorylation of cellular proteins, including ERK.

Gas6/Tyro 3 signaling in osteoclasts

In a pit formation assay on a dentine slice resorbed by crude osteoclastic cells formed in the coculture of mouse osteoblasts and bone marrow cells, Gas6 dose-dependently stimulated the resorbed pit area. This stimulation was not due to the increase in the number of osteoclasts, but to the activation of each osteoclast function because the number of thrombospondin-related anonymous protein (TRAP)-positive multinucleated osteoclasts on a dentine slice was not affected by Gas6. In fact, the effect of Gas6 on the pit area per osteoclast (resorbed pit area/osteoclast number in a dentine slice) showed a similar pattern to that on the resorbed pit area.

To study the expression of Gas6 and Tyro 3 in cells of osteoblastic and osteoclastic lineages, mRNA levels in osteoblasts, osteoclasts, spleen cells, bone marrow cells, and brain were examined by reverse transcriptase (RT)-PCR. Gas6 was expressed in all cells examined, but the expression of Tyro 3 was detected only in osteoclasts and brain. To investigate the expression of Tyro 3 during the differentiation of osteoclastic cells, Tyro 3 mRNA levels were examined in spleen cells cultured in the presence of soluble RANKL and M-CSF without the support of osteoblastic/stromal cells. TRAP-positive multinucleated osteoclasts became detectable at 5 days of culture and had increased at 6 days, and Tyro 3 expression was detected slightly at 5 days and was abundant at 6 days of culture. These findings confirm that Tyro 3 is expressed predominantly in mature osteoclasts but not in osteoclast precursors. We therefore suggest that this localization of Tyro 3 may explain the selective action of Gas6 on the function of mature osteoclasts.

To learn the mechanism of Gas6/Tyro 3 signaling in mature osteoclasts, we examined the time course of the effects of Gas6 on tyrosine phosphorylation of cellular proteins in isolated mouse osteoclasts (Fig. 2A). Several proteins were phosphorylated by Gas6 as early as 1 min, and this activation was maintained for 10 min. Western blot analyses using antibodies against specific proteins related to

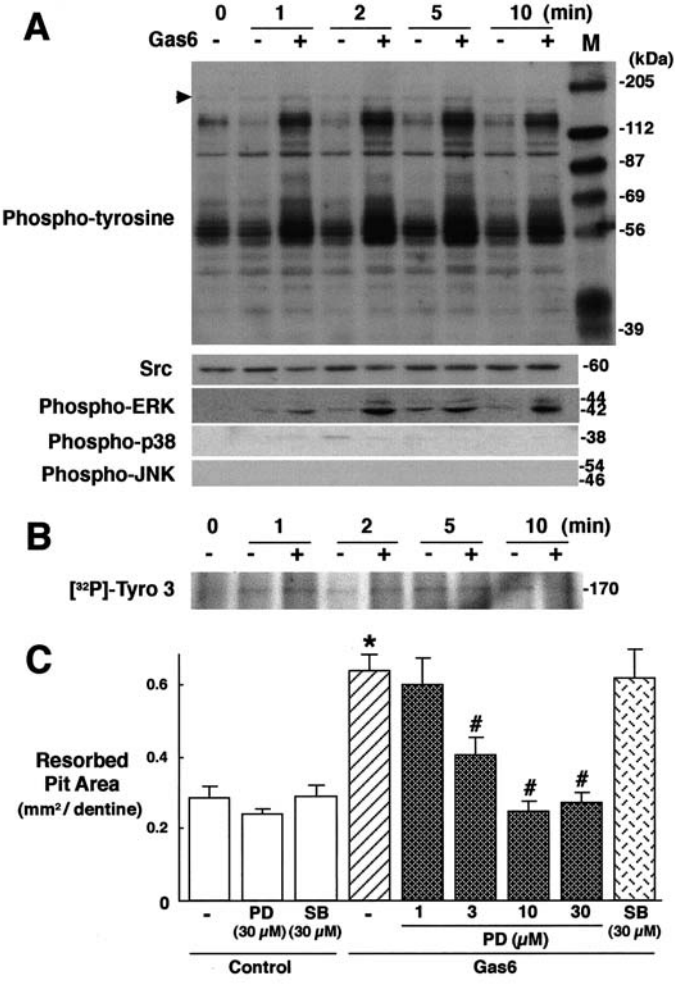

Fig. 2. Intracellular signaling through Gas6/Tyro 3 in isolated osteoclasts. **A** Effects of Gas6 on phosphorylation of cellular proteins and MAPKs in isolated osteoclasts. Mouse osteoclasts isolated from the coculture were cultured with or without Gas6 (5×10^{-9} M) for the period indicated (0–10 min) and lysed with TNE buffer. Twenty μg cell lysates was subjected to 7.5% SDS–PAGE, and immunoblotted with antibodies against phosphotyrosine, src, phospho-ERK, phospho-p38 MAPK, and phospho-JNK MAPK. The *arrow head* indicates 170 kD protein (the same size as Tyro 3). **B** Tyrosine kinase activity of immunoprecipitated Tyro 3 in isolated osteoclasts. Isolated osteoclasts were cultured with and without Gas6 (5×10^{-9} M) for various periods (1–10 min), lysed with TNE buffer, and 100 μg of cell lysates was immunoprecipitated with polyclonal anti-Tyro 3 antibody. The samples were incubated in kinase buffer with [g-^{32}P]-ATP, and subjected to SDS–PAGE. **C** Effects of PD98059 (PD) and SB203580 (SB) on resorbed pit formation stimulated by Gas6 on a dentine slice. Osteoblasts from neonatal mouse calvariae and bone marrow cells from 8-week-old mice were co-cultured on a collagen gel to form osteoclasts. Crude osteoclastic cells released from the co-culture on a collagen gel were further cultured on a dentine slice with or without Gas6. Gas6 (10^{-8} M), PD98059 (1, 3, 10, and 30 μM), and SB203580 (30 μM) were added to the culture 1 h after seeding. After 48 h culture, the total pit area in a dentine slice was measured. Data are expressed as means (bars) ± SEMs (error bars) for eight cultures/group. *$P < 0.01$, significant stimulation by Gas6; #$P < 0.01$, significant inhibition by PD98059

MAPKs revealed that phosphorylation of ERK was seen at 1 min and maintained for 10 min, while neither p38 nor JNK MAPK phosphorylation was seen (Fig. 2A). To investigate the autophosphorylation of Tyro 3 by Gas6, the kinase ac-

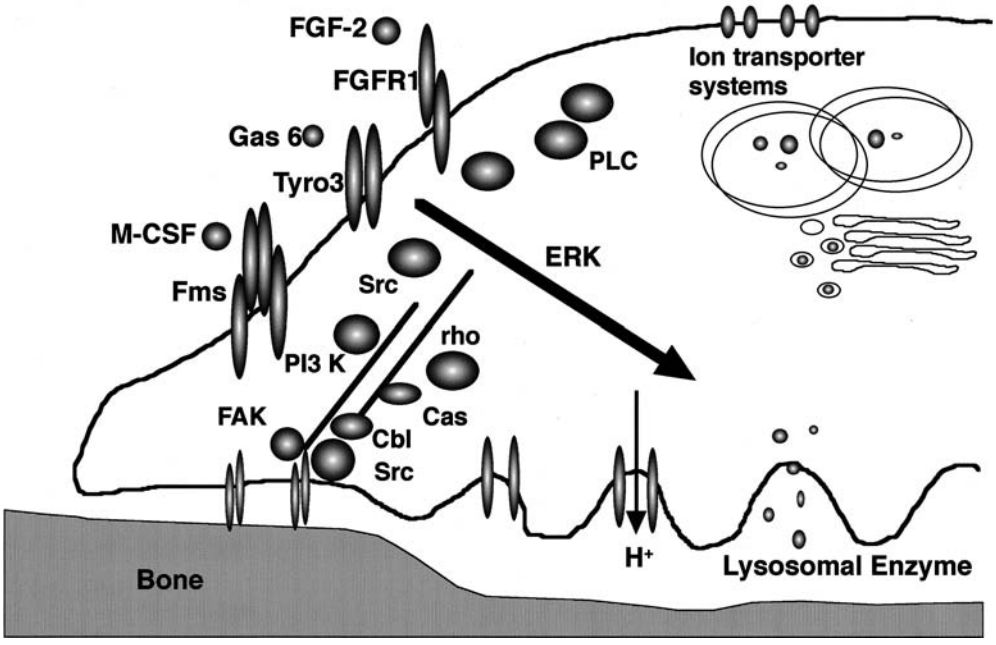

Fig. 3. Scheme showing the regulation of osteoclast activation by RTK signaling in osteoclasts

tivity of immunoprecipitated Tyro 3 was examined by in vitro kinase assay (Fig. 2B). Gas6 induced the kinase activity of Tyro 3 at 1 min, which reached a maximum at 2 min, and had decreased considerably after 5 min. To examine the functional relevance of the activation of ERK by Gas6 in osteoclasts, PD98059 was added to the pit formation assay system (Fig. 2C). PD98059 (1–30 μM) dose-dependently inhibited the stimulation of Gas6 on pit formation resorbed by mouse osteoclasts to the levels of the control culture, while SB203580 (30 μM) did not affect the Gas6 stimulation. We therefore conclude that Gas6 stimulated osteoclast bone resorptive function depending on the restricted localization of its receptor Tyro 3 on mature osteoclasts. Gas6 was further shown to activate osteoclasts through the phosphorylation of Tyro 3 and ERK in mature osteoclasts.

Discussion

We have found that FGF-2 and Gas6 act directly on mature osteoclasts through the activation of FGFR1 and Tyro 3, respectively, by way of ERK phosphorylation[6,11] (Fig. 3). M-CSF is also reported to activate another RTK c-Fms, followed by the activation not only of the ERK pathway,[10] but also of the c-src-dependent pathway.[8] c-Src, a ubiquitous cellular tyrosine kinase which is strongly expressed in osteoclasts, is essential for osteoclasts to form a ruffled border and to resorb bone,[16] and the contribution of c-src kinase to RTK signaling has been suggested.[17] In our studies, inhibitors of the src family kinases, herbimycin and PP1, abrogated the osteoclast function in control cultures as well as in FGF-2- or Gas6-stimulated cultures (data not shown). Hence, we assume that the src kinase signal may be essential for the basal osteoclast function, while ERK is the major pathway for the RTK signaling.

Although the essential component of signaling regulating osteoclast differentiation and function is controversial, the roles of nuclear factor (NF)-κB and JNK have been extensively investigated.[18] The stimulation by RANKL has been shown to be mediated by the activation of NF-κB, which is dependent on the interaction with tumor necrosis factor receptor-associated factor 6 (TRAF6) or TRAF2.[19] In fact, knockout mice of both NF-κB1 and NF-κB2, and of TRAF6 exhibited severe osteopetrosis due to impaired osteoclast differentiation and function.[20,21] Although RANKL also activates JNK in osteoclasts,[18] the role of osteoclast function is still controversial. In this study, JNK does not seem to mediate the FGF-2 or Gas6 effect judging from the lack of JNK phosphorylation by these cytokines. The essential signal pathway for the survival of osteoclasts is also controversial. The NF-κB pathway[9] and the ERK pathway[10] have been reported to be important in sustaining the survival of osteoclasts. In the present study, although FGF-2 and Gas6 induced the activation of ERK, this did not promote the survival of osteoclasts.[6,11] However, this result cannot rule out the possibility of the involvement of ERK in osteoclast survival because ERK activation by these cytokines might be insufficient to sustain the survival, while being sufficient to activate osteoclast function.

Joint destruction in rheumatoid arthritis (RA) patients is also accompanied by the acceleration of osteoclastic bone resorption. We recently reported that increased FGF-2 in the synovial fluid contributed to joint destruction through direct action on mature osteoclasts.[22] Expressions of Gas6 and Tyro3 have recently been demonstrated in the synovium of RA patients.[23] These observations suggest that Gas6 as well as FGF-2 play some role in the pathological

bone disorders in these diseases. In addition, Gas6 and FGF-2 have a common action in the induction of angiogenesis. Regarding angiogenic growth factors other than Gas6 and FGF-2, vascular endothelial growth factor (VEGF) has been reported to enhance osteoclastic bone resorption directly and indirectly in cultures.[23–26] These findings indicate that several angiogenic factors have a common action in stimulating osteoclastic function as well as angiogenesis, suggesting a positive interrelationship between bone resorption and the invasion of blood vessels during bone modeling and remodeling.

The results in these studies show that RTK signaling may play an important role in mature osteoclast function. We now suggest the possibility that signaling through RTKs on osteoclasts contributes not only to osteoclastic function, but also to the pathophysiology of osteopenic disorders such as joint destruction in RA.

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