YY1 is involved in RANKL-induced transcription of the tartrate-resistant acid phosphatase gene in osteoclast differentiation

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Abstract

Receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL), a critical activator of osteoclast differentiation, plays a pivotal role in tartrate-resistant acid phosphatase (TRAP) gene expression. Previously, we showed that upstream stimulatory factors (USF) 1 and 2 are implicated in the RANKL-induced TRAP transcriptional activation via a 12-bp USF binding site in the TRAP promoter. In that study, we also demonstrated that a RANKL-induced nuclear protein binds to a 50-bp oligonucleotide (Oligo IV) corresponding to a distinct TRAP promoter region. Here we report the identification and functional characterization of the nuclear protein binding to Oligo IV. We identified a 21-bp sequence CTGTTTATGATGGCGAGGGGG in Oligo IV that specifically binds the RANKL-induced nuclear protein from RAW264.7 cells by performing a series of competition assays. Computer analysis of the 21-bp sequence revealed that the sequence contains a putative Yin Yang 1 (YY1) binding site overlapped with a putative activator protein-2 (AP-2) binding site. Competition and supershift assays indicated that the nuclear protein binding to the 21-bp sequence is YY1, not AP-2. Functionally, mutation of the YY1-binding site resulted in a reduction in the RANKL-induced TRAP transcription in RAW264.7 cells, demonstrating that YY1 positively regulates RANKL-induced TRAP transcriptional activation. In conclusion, our data demonstrated that YY1 plays a functional role in RANKL-mediated TRAP gene expression during osteoclast differentiation.

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1. Introduction

Osteoclasts are multinucleated bone-resorbing cells that play an important role in both development and maintenance of the skeleton. Osteoclasts differentiate from mononuclear precursors of monocyte/macrophage lineage upon stimulation of two critical factors: monocyte/macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) (Teitelbaum, 2000). It has been shown that a number of genes including those encoding tartrate-resistant acid phosphatase (TRAP), calcitonin, integrin β3 subunit, c-Src and cathepsin K are activated during osteoclastogenesis (Hsu et al., 1999). Furthermore, TRAP, calcitonin, integrin β3 subunit, c-Src and cathepsin K are all functionally implicated in osteoclastic bone resorption (Feng et al., 2001; Gelb et al., 1996; Hayman et al., 1996; Soriano et al., 1991; Zaidi et al., 1989).

Among these proteins, TRAP has drawn special attention from bone biologists. TRAP expression is barely detectable in osteoclast precursors, but its expression is dramatically increased during osteoclastogenesis (Oddie et al., 2000).
Thus, it has been widely used as a marker for osteoclast differentiation (Teitelbaum, 2000). More importantly, consistent with its abundant expression in mature osteoclasts, TRAP plays a functional role in bone resorption (Yaziji et al., 1995). A neutralizing antibody against TRAP inhibited bone resorption in vitro (Zaidi et al., 1989). Moreover, mice lacking TRAP exhibited a defect in endochondral ossification and a mild osteoporosis while transgenic mice over-expressing TRAP showed a decrease in trabecular bone density with the characteristics of a mild osteoporosis (Angel et al., 2000; Hayman et al., 1996). It was shown that TRAP may regulate bone resorption by mediating the degradation of endocytosed matrix products during transcytosis in activated osteoclasts (Halleen et al., 1999; Nesbitt and Horton, 1997).

RANKL is a member of the tumour necrosis factor (TNF) superfamily (Lacey et al., 1998), and is a critical regulator of osteoclast differentiation, function and survival (Hsu et al., 1999; Wong et al., 1997). It has been well established that RANKL activates two critical intracellular signalling pathways: nuclear factor kappa B (NF-κB) (Darnay et al., 1998; Hsu et al., 1999) and c-Jun N-terminal kinase (JNK) (Darnay et al., 1998; Hsu et al., 1999). These two pathways play an essential role in osteoclast differentiation and function (Franzoso et al., 1997; Grigoriadis et al., 1994). In addition, it has been recently shown that nuclear factor of activated T cells (NFATc1) is also implicated in RANKL-mediated osteoclastogenesis (Takayanagi et al., 2002).

Several lines of evidence indicate that RANKL plays a key role in TRAP expression during osteoclast differentiation (Lacey et al., 1998). We previously investigated the molecular mechanism underlying the RANKL-mediated TRAP expression during osteoclast differentiation and identified an upstream stimulatory factor (USF) binding sequence in the TRAP promoter mediating TRAP transcriptional activation in response to RANKL (Liu et al., 2003). In the course of that study, we also found that a RANKL-induced nuclear protein binds to an oligo (designated as Oligo IV) corresponding to a TRAP promoter region from −1124 to −1074 in electrophoretic mobility shift assay (EMSA) (Liu et al., 2003). In the present study, we elucidated the specific nuclear protein binding sequence in Oligo IV and showed that the nuclear protein binding to the site is Yin Yang 1 (YY1). More significantly, we demonstrated that YY1 is functionally involved in the regulation of RANKL-dependent transcriptional activation of TRAP gene.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Synthetic oligonucleotides (oligos) were purchased from Sigma-Genosys (The Woodlands, TX). Antibodies used for supershift assays, including c-Fos (sc-52X), c-Jun (sc-44X), p65 (sc-372X), p50 (sc-1190X) and YY1 (sc-281X), were from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Cell culture and transient transfections

RAW 264.7 cells were purchased from ATCC (Manassas, VA) and cultured in DMEM containing 10% heat-inactivated FBS in tissue culture plates and passed by lifting the cells by scraping. Transient transfections were performed using LipofectAmine Plus transfection reagents from Invitrogen (Carlsbad, CA). One day prior to transfections, RAW 264.7 cells were plated in 6-well cell culture plates at 3×10^5 cells/well. For each well, 2 μg reporter plasmid plus 0.05 μg internal control plasmid phRL-SV40 (Promega, Madison, MI) were used. Transfected cells were treated with or without 200 ng/ml GST-RANKL (Liu et al., 2003) for 4 days after transfection and lysed for luciferase assays using Dual-Luciferase Reporter Assay System from Promega.

2.3. Nuclear extract preparation

Nuclear extracts were prepared as described in Liu et al. (2003). Briefly, upon confluence (about 1×10^7 cells), cells were washed three times with cold phosphate-buffered saline (PBS) and then scraped off the dishes. Cells were centrifuged at 16,000×g for 5 min and resuspended in 500 μl of Hypotonic Lysis Buffer [10 mM HEPES–KOH, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. DTT and PMSF were added immediately prior to use. Cells were lysed for 15 min on ice, at which time 32 μl of 10% Nonidet P-40 was added to the suspension, followed by vortexing the tube for 15 s and incubating on ice for 10 min. Nuclei were spun down and nuclear extracts were prepared by resuspending and incubating the nuclei in 100 μl of Nuclear Extraction Buffer [20 mM HEPES–KOH, pH 7.9, 420 mM NaCl, 1.2 mM MgCl2, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM PMSF, 0.5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 5 μg/ml pepstatin, and 5 μg/ml leupeptin]. DTT, PMSF, AEBSF, pepstatin, and leupeptin were added immediately prior to use. Protein concentrations were determined using the BioRad Protein Assay (Hercules, CA).

2.4. Electrophoretic mobility shift assays (EMSA)

Oligos were end-labelled with 32P using T4 polynucleotide kinase (Invitrogen). A 2–5×10^4 cpm probe was incubated with 3 μg of nuclear extracts in a 20-μl volume of binding reaction [10 mM Tris–Cl, pH7.5, 100 mM NaCl, 10% glycerol, 50 ng/ml poly(dI/dC)] on ice for 20 min. For competition assays, a 50-fold excess amount of unlabeled...
competitors was premixed with labelled probe before being added to the binding mixture. The binding reaction was incubated for 20 min on ice. For supershift assays, probe was incubated with 3 μg nuclear extracts in a 20-μl volume of binding reaction for 20 min on ice, at which time 4 μg control IgG or 4 μg specific antibodies were added, followed by incubation on ice for an additional 30 min. Reaction mixtures were resolved on 4–20% gradient gels (Invitrogen) using 0.5× TBE running buffer at 4 °C and 100 V for 3.5 h in a Novex Xcell II (Invitrogen) minicell electrophoresis system. The gels were transferred to 3 M blotting paper, dried and exposed to film (Kodak BioMax MS films).

2.5. Site-directed mutagenesis

Point mutations were introduced in the context of the longest TRAP promoter construct TP(−1858) using a QuickChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers (oligos) used to mutate the YY1 binding site are 5'-AAAGGCAGTTATT-GCTGCTGTATTAGATttaGAGGGGGAACT-CGG-3' and 5'-CCGAGTTCCCCCCTtaaATCATAAA-CAGCAGCAAT- AAATCCTT-3. These oligos were purified by polyacrylamide gel electrophoresis. PCR reactions were performed according to the manufacturer’s protocol. The

Fig. 1. Location and sequence of the TRAP promoter region corresponding to Oligo IV. (A) Schematic location of the 200-bp TRAP promoter region (−1239 to −1039, relative to the translation start site) that was previously shown to regulate RANKL-induced TRAP transcriptional activation (Liu et al., 2003). (B) Schematic diagram showing five overlapping oligos (Oligos I, II, III, IV and V) spanning the entire 200-bp region mediating RANKL-induced TRAP transcription. In particular, Oligos I and IV bound RANKL-activated nuclear proteins from RAW264.7 cells (Liu et al., 2003). (C) Nucleotide sequence of Oligo IV.

Fig. 2. Oligo IV binds transcription factors other than NF-κB and AP-1. (A) Competition assays. Competition assays were performed using labelled oligo IV as probe and nuclear extracts (N.E.) from RAW264.7 cells treated with RANKL (200 ng/ml) for 4 days: RAW(RANKL). Excess (50×) cold oligo IV (lane 2), NF-κB oligo (lane 3) and AP-1 oligo (lane 4) were added as competitors (Comp). As control, no competitor was added in lane 1 (None). NF-κB oligo and AP-1 oligo are from Santa Cruz Biotechnology. (B) Supershift assays. Supershift assays were performed using labelled oligo IV as probe and nuclear extracts (N.E.) from RAW264.7 cells treated with RANKL (200 ng/ml) for 4 days: RAW(RANKL). No antibody was added in lane 1 (None). Different antibodies were added in lanes 2–6: IgG in lane 2 as control; p50 in lane 3; p65 in lane 4; c-fos in lane 5 and c-jun in lane 6.
PCR products were treated with DpnI (10 units) for 60 min at 37 °C. XL1-blue supercompetent cells were transformed with the DpnI-treated PCR mixture as described in the instruction manual and plated on ampicillin plates. Plasmids were prepared from individual colonies and sequenced to confirm the mutations.

2.6. Sequence analysis

Sequence analysis was performed using the Genetic Computer Group (Madison, WI) sequence analysis software.

3. Results

3.1. Oligo IV binds transcription factors other than NF-κB and AP-1

In our previous study, we demonstrated that a 1.8-kb mouse TRAP promoter contains two distinct promoter regions (one at −1858 to −1239 and the other at −1239 to −1039, relative to the translation start site) that are independently capable of mediating RANKL-induced TRAP promoter activation (Liu et al., 2003). Further studies with five overlapping oligos (Oligo I, II, III, IV and V) showed that Oligo I and Oligo IV bind RANKL-induced nuclear proteins from RAW 264.7 cells (Liu et al., 2003). A detailed characterization of Oligo I and its associated nuclear proteins led to the identification of a USF binding site AGCCACGGTTG (−1209 to −1198) in the TRAP promoter that regulates RANKL-induced TRAP promoter activation (Liu et al., 2003). In the present study, we seek to characterize the specific sequence in Oligo IV and its associated nuclear protein that mediate the RANKL-induced TRAP promoter activation. Fig. 1 illustrates the schematic location of the region at −1239 to −1039 in the 1.8-kb TRAP promoter (Fig. 1A) as well as location and sequence of Oligo IV (Fig. 1B and C).

First, we determined whether the binding of the nuclear protein to Oligo IV is specific by performing EMSA/competition assays using excess cold Oligo IV (Fig. 2A). While Oligo IV bound a nuclear protein from RANKL-treated RAW264.7 cells (lane 1, Fig. 2A), the excess cold Oligo IV was able to compete for the nuclear protein binding (lane 2, Fig. 2A), indicating that the binding is specific. Furthermore, it has been well established that RANKL

Fig. 3. Identification of a 21-bp sequence binding the nuclear protein in Oligo IV using competition assays. (A) Diagram of shortened oligos IV used for competition assays. The identified 21-bp sequence is shown in bold. Two broken lines highlight the 5’- and 3’-ends of the sequence. (B) Localization of the 5’- and 3’-ends of the sequence binding the RANKL-induced nuclear protein. Competition assays were performed using labelled oligo IV as probe and SOIV 1–8 as competitors (Comp). Nuclear extracts (N.E.) used for the assays were from RAW264.7 cells treated with RANKL (200 ng/ml) for 4 days: RAW(RANKL).
activates two important transcription factors NF-κB and activator protein-1 (AP-1) (Daray et al., 1998; Galibert et al., 1998; Hsu et al., 1999) and both NF-κB and AP-1 play a pivotal role in osteoclastogenesis (Franzoso et al., 1997; Grigoriadis et al., 1994). Thus, we searched for potential binding sequences for NF-κB and/or AP-1 in Oligo VI and the search indicated that Oligo VI contains neither NF-κB nor AP-1 sequence. We next examined whether the nuclear protein binding to Oligo IV is NF-κB or AP-1. To this end, we used excess cold oligos containing NF-κB/AP-1 consensus sequences as competitors to perform EMSA/competition assays (Fig. 2A). We found that both NF-κB and AP-1 oligos failed to compete for the nuclear protein binding (lanes 3 and 4, Fig. 2A), suggesting that the nuclear protein binding to Oligo IV is neither NF-κB nor AP-1. We also performed supershift assays with antibodies against p50 or P65 (the components of NF-κB) and those against c-fos or c-jun (the components of AP-1) (Fig. 2B). In our positive controls, these antibodies effectively bound these transcription factors (data not shown). However, the supershift assays demonstrated that none of the four antibodies was able to supershift the band (lanes 3, 4, 5, and 6, Fig. 2B), further indicating that Oligo IV binds transcription factors other than NF-κB and AP-1 (Fig. 2B).

3.2. Identification of a 21-bp sequence CTGTTTATGATGGCGAGGGGG that specifically binds the RANKL-induced nuclear protein in Oligo IV

We decided to identify the specific nuclear protein binding sequence in Oligo IV as the first step in characterizing the nuclear protein binding to Oligo IV. In doing so, we performed a series of EMSA/competition assays using eight shortened oligos (SOIVs 1, 2, 3, 4, 5, 6, 7 and 8) derived from Oligo IV as competitors (Fig. 3A). SOIVs 1, 2, 3 and 4 have the same 5′ end but different 3′ ends. In contrast, SOIVs 5, 6, 7 and 8 start at different 5′ positions, but they end at the same 3′ position. As shown in Fig. 3B, while SOIV 1 was still able to displace the nuclear protein binding, SOIVs 2–4 were ineffective competitors, revealing the 3′ end of the specific nuclear protein binding sequence (Fig. 3A). Similarly, SOIVs 5 and 6 could compete efficiently, but SOIVs 7 and 8 did not compete for the binding, elucidating the 5′ end of the sequence (Fig. 3A). Taken together, these assays identified a 21-bp sequence CTGTTTATGATGGCGAGGGGG within the 50-bp region that specifically binds the nuclear protein induced by RANKL.

3.3. The nuclear protein binding to Oligo IV is YY1

Upon identification of the 21-bp nuclear protein binding sequence, we performed computer analysis (FindPattern program, Genetic Computer Group) to search for putative transcription factor binding sites in this sequence. The computer analysis revealed that the 21-bp sequence contains putative binding sequences for two transcription factors: YY1 (Shi et al., 1997; Thomas and Seto, 1999) and activator protein-1 (AP-2) (Hilger-Eversheim et al., 2000) (Fig. 4A). To determine whether one or both of the transcription factors bind the sequence, we performed competition assays with excess cold oligos containing YY1 or AP-2 consensus sequence. As shown in Fig. 4B, while the oligo containing YY1 consensus sequence competed efficiently for the nuclear protein binding (lane 3, Fig. 4B), the one containing AP-2 consensus sequence failed to compete for the nuclear protein binding (lane 4, Fig. 4B), suggesting that the nuclear protein binding to Oligo IV is YY1. This finding was confirmed by our supershift assay showing that an antibody against YY1 blocked the binding of the nuclear protein to Oligo IV (lane 3, Fig. 4C). Notably, while the band disappeared with the addition of the antibody, there is apparent lack of a supershifted band (lane 3, Fig. 4C). This data indicate that the binding of the antibody to YY1 disrupted the interaction of YY1 with the labeled Oligo IV (resulting in an immune complex containing the antibody and YY1 only) rather than formed a bigger complex (antibody/YY1/labeled Oligo IV) which usually exhibits a slower mobility in EMSA. Nonetheless, the data in Fig. 4C demonstrated that the antibody specifically recognized YY1, confirming that the nuclear protein binding to Oligo IV is YY1.
3.4. The 21-bp YY1 binding sequence is functionally involved in the RANKL-induced TRAP transcription

To determine whether the 21-bp YY1-binding sequence plays a functional role in the RANKL-dependent TRAP transcription, we first identified a mutation capable of abrogating the YY1 binding. As shown in Fig. 5A, we synthesized two mutant Oligo IV (mOligo 1 and mOligo 2). In mOligo 1, GGC, in the core YY1 binding sequence TGGCGA, was converted to AAT (Fig. 5A). In mOligo 2, GGC, in the core YY1 binding sequence TGGCGA, was converted to TTA (Fig. 5A). EMSA with these mutant oligos showed that both mOligo 1 and mOligo 2 failed to bind YY1 (lanes 2 and 3, Fig. 5B), indicating that the introduced mutation in both mutant oligos are sufficient to eliminate the binding capacity of the sequence. However, while mOligo 1 failed to bind YY1, it gained unwanted capacity to bind other nuclear proteins with considerable affinity (lane 2, Fig. 5B). In contrast, mOligo 2 not only failed to bind YY1 but it also did not bind any other nuclear proteins (lane 3, Fig. 5B). Based on these data, we chose to use the mutation in mOligo 2 rather than the one in mOligo 1 for our subsequent studies described below.

We introduced the same mutation in mOligo 2 in our longest reporter construct TP(−1858) (Liu et al., 2003), resulting in a reporter construct named Mutant (Fig. 6A). To determine whether the mutation of YY1 site affects the RANKL-induced TRAP promoter activation, we transfected RAW264.7 cells with the mutant and the wild-type construct TP(−1858) using Lipofectamine as previously described (Liu et al., 2003). However, in the current experiments, we further optimized transfection efficiencies by using lower cell density and these conditions resulted in higher promoter activation by RANKL (data not shown). As shown in Fig. 6B, the data from three independent sets of the transfection studies indicated that TP(−1858) resulted in 4.9-fold induction in response to RANKL, but Mutant gave rise to only 3.5-fold induction ($p<0.01$). Thus, we conclude that the 21-bp YY1 binding sequence is positively involved in RANKL-induced transcriptional activation of TRAP gene.

4. Discussion

TRAP is not only abundantly expressed in mature osteoclasts, but it also plays an important role in osteoclastic bone resorption (Angel et al., 2000). Since the unravelling of the RANKL/RANK system in the late 1990s, it has been well established that RANKL plays a pivotal role in TRAP expression during osteoclast differentiation (Lacey et al., 1998). However, the molecular mechanism by which RANKL activates the TRAP gene expression during osteoclast differentiation is not completely understood. We reasoned that the investigation of the molecular mechanism
of RANKL-mediated TRAP gene expression during osteoclast differentiation will not only provide a better understanding of TRAP function in osteoclasts but may also elucidate novel transcriptional mechanisms by which RANKL controls gene expression.

In our previous study, we investigated the molecular mechanism by which RANKL regulates TRAP expression during osteoclast differentiation by characterizing a 1.8-kb mouse TRAP promoter (Liu et al., 2003). This study revealed that USF proteins are implicated in the RANKL-mediated TRAP gene transcription by binding to a USF site located at \(-1180\) to \(-1169\), relative to translation start site (Liu et al., 2003). Interestingly, we also noticed that a 50-bp oligo used for EMSA (Oligo IV, corresponding to the TRAP promoter region from \(-1124\) to \(-1074\)) bound a nuclear protein induced by RANKL. In the present study, we extended the previous work to investigate whether the RANKL-activated nuclear protein binding to Oligo IV is involved in RANKL-dependent TRAP transcription. Using a series of assays such as EMSA, competition assays and supershift assays, we not only identified a 21-bp sequence CTGTTTATGATGGCGAGGGGG that is required for the nuclear protein binding, but, more significantly, we also established that this 21-bp sequence is involved in RANKL-induced TRAP transcriptional activation by binding the transcription factor YY1. Consistently, there is considerable homology in the 21-bp sequence between human and mouse. The evolutionary conservation further supports the functional role of YY1 in TRAP gene expression in osteoclasts.

YY1 is a multifunctional transcription factor that can act as a transcriptional repressor, an activator, or an initiator element-binding protein (Flanagan et al., 1992). YY1 belongs to the GLI-Kruppel family of zinc finger transcription factors (Thomas and Seto, 1999) and is highly conserved among human, mouse and \textit{Xenopus} (Shi et al., 1997). The C-terminal domain of YY1 consists of four C2H2 zinc fingers that bind the core DNA sequence CGCCATNTT, which is found in numerous gene promoters (Thomas and Seto, 1999). Many of YY1 target genes are implicated in regulating cell growth and differentiation (Shi et al., 1997; Thomas and Seto, 1999). YY1 has been shown to regulate the expression of several genes such as osteocalcin and Msx2 in osteoblasts (Paredes et al., 2002; Tan et al., 2002). Of particular interest, a recent study showed that YY1 is involved in signalling by TNF\(\alpha\) (Kalayci et al., 2003), which regulates osteoclast differentiation and function (Teitelbaum, 2000). Given that TRAP gene expression is increased during osteoclast differentiation and TRAP plays a role in osteoclastic bone resorption, it is reasonable to suggest that YY1 may be an important transcription factor involved in osteoclast differentiation and bone remodelling. However, since YY1 knockout mice are embryonic lethal (Donohoe et al., 1999), the role of YY1 in
the differentiation of these two important bone cells and subsequently bone remodelling in vivo still remains unknown.

YY1 regulates gene transcription primarily by interacting with other transcription activators or repressors binding to regulatory sequences in the promoter of its target genes (Shi et al., 1997; Thomas and Seto, 1999). It was well established that YY1 interacts with a number of key regulatory transcription factors such as TBP, TFIIB, TAFII55, Sp1, Myc and E1A (Thomas and Seto, 1999)(Shrivastava et al., 1996). Consistent with this notion, YY1 alone is not able to activate TRAP transcription in osteoclast differentiation since we have previously shown that TRAP promoter deletion mutants such as TP(-1199), TP(-1159), TP(-1119) (Liu et al., 2003), which all still have the YY1 binding site, failed to mediate TRAP transcription activation in response to RANKL (summarized in Fig. 7). Thus, it is likely that YY1 regulates RANKL-induced TRAP transcription by interacting with transcription factors binding to other regions of the TRAP promoter. Given that TRAP promoter deletion mutants such as TP(-1199), TP(-1159) and TP(-1119) failed to mediate RANKL-induced TRAP transcription activation (Liu et al., 2003), it is reasonable to hypothesize that YY1 modulates the TRAP transcription activation by interacting with the transcription factors binding to the sequences upstream of the YY1 site (Fig. 7). Previously, we have shown that USF1 and USF2 are implicated in regulating RANKL-induced TRAP promoter activation. Whether YY1 interacts with USF proteins in the regulation of TRAP promoter activity remains to be investigated. Alternatively, as we demonstrated in our previous study (Liu et al., 2003), the TRAP promoter region (−1858 to −1239), which is upstream of the USF binding sequence, is also involved in the regulation of RANKL-induced TRAP promoter activation. Thus, the other possibility is that YY1 regulates RANKL-induced TRAP promoter activation through interacting with the transcription factors binding to this region. We are currently investigating the cis-element(s) located in this region (−1858 to −1239) and the corresponding trans-element(s) that mediates RANKL-induced TRAP promoter activation. Once the cis-element(s) and the trans-element(s) are identified, we will explore this possibility.

Our transfection studies demonstrated that the mutation of the YY1-binding site in the longest reporter construct TP(−1858) only gave rise to a partial reduction (about 28%) in the RANKL-mediated TRAP promoter activation (Fig. 6). Previously, we showed that two distinct promoter regions (one at −1858 to −1239 and the other at −1239 to −1039, relative to the translation start site) are independently capable of mediating RANKL-induced TRAP promoter activation (Liu et al., 2003), suggesting that multiple transcription factors may be implicated in the RANKL-dependent TRAP transcriptional activation. As such, it is expected that the inhibition of one of the transcription factors involved in the RANKL-induced TRAP transcriptional activation may only lead to a partial reduction in the transcriptional activation.

Interestingly, a recent study revealed that three tissuespecific promoters regulate the transcription of the murine TRAP gene (Walsh et al., 2003). These promoters are located at different regions (the first one: −5714 to −5582; the second one: −1837 to −1728; the third one: −649 to −495) upstream of the translation start site, which is designated as +1. These promoters can independently serve as basal transcription units that are capable of initiating transcription, thus giving rise to TRAP mRNA with different 5′ ends (Walsh et al., 2003). In particular, the study

![Fig. 7. Model on action of YY1 in RANKL-induced TRAP transcriptional activation. Schematic location of the YY1-binding site is shown in TP(−1858). As shown in Fig. 6, the mutation of the YY1 site in TP(−1858) led to a reduction in RANKL-induced TRAP promoter activation. However, the previous studies demonstrated that the deletion constructs such as TP(−1199), TP(−1159) and TP(−1119), which still contain the YY1 site, failed to mediate TRAP transcription activation in response to RANKL (Liu et al., 2003). Thus, these data suggest that YY1 mediates RANKL-induced TRAP promoter activation in collaboration with transcription factors that bind to the promoter sequences upstream of the YY1 site.](image-url)
showed that the expression of TRAP in osteoclasts is primarily mediated by the third promoter (the one at −649 to −495). As discussed above, our previous work identified two distinct TRAP promoter regions (one at −1858 to −1239 and the other at −1239 to −1039) that are able to mediate RANKL-dependent activation of the TRAP gene during osteoclastogenesis. However, these two regions are different from the three promoters described by Walsh et al. (2003) that in they contain RANKL-responsive cis-elements rather than serve as basal transcription units. Thus, the RANKL-responsive cis-elements in these two regions and the corresponding transcription factors are most likely to activate the TRAP transcription during osteoclastogenesis through the basal transcription machinery involving the third promoter identified by Walsh et al. (2003).

In conclusion, the data demonstrates that YY1 plays a functional role in RANKL-dependent TRAP transcription. The findings provide more insights into the molecular mechanism by which RANKL regulates the transcription of TRAP gene. Nonetheless, this study also raises many important questions. First, the transcription factor(s) that YY1 interacts with while regulating TRAP transcription are unknown. In addition, besides its established role in regulating TRAP gene expression in osteoclasts, whether YY1 is involved in osteoclast differentiation and bone remodelling in vivo remains unanswered. Future studies aimed at addressing these questions will elucidate novel insights into molecular mechanisms governing osteoclast differentiation and function.

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