Involvement of Upstream Stimulatory Factors 1 and 2 in RANKL-Induced Transcription of Tartrate-Resistant Acid Phosphatase (TRAP) Gene during Osteoclast Differentiation

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Running Title: Transcriptional Regulation of TRAP Gene by RANKL
SUMMARY

Tartrate-resistant Acid Phosphatase (TRAP) plays an important role in bone resorption. TRAP expression in osteoclasts is regulated by RANKL, a potent activator of osteoclast differentiation. However, the molecular mechanism underlying the RANKL-induced TRAP expression remains unknown. Here we show that two regions in the mouse TRAP promoter (one at –1858 to -1239 and the other at –1239 to -1039, relative to the translation start site) are implicated in RANKL-induced TRAP transcription in RAW264.7 cells. A detailed characterization of the region at –1239 to –1039 identifies a 12-bp sequence AGCCACGTGGTG that specifically binds nuclear proteins from RAW264.7 cells and primary bone marrow macrophages (BMMs) in EMSA. Moreover, the binding is significantly enhanced in EMSA with nuclear extracts from RANKL-treated RAW264.7 cells and BMMs, suggesting that the 12-bp sequence may be involved in RANKL-induced TRAP transcription. Various assays reveal that nuclear proteins binding to the 12-bp sequence are upstream stimulatory factors (USF) 1 and 2. Importantly, mutation of the USF-binding site partially blocks RANKL-induced TRAP transcription in RAW264.7 cells, confirming that USF1 and USF2 are functionally involved in RANKL-induced TRAP transcription. In summary, our data show that USF1 and USF2 play a functional role in RANKL-dependent TRAP expression during osteoclast differentiation.
INTRODUCTION

Osteoclasts, the principal bone-resorbing cells, play a pivotal role in skeletal development and maintenance (1). Osteoclasts are derived from mononuclear precursors of monocyte/macrophages lineage upon stimulation of two critical factors: M-CSF and RANKL (2). Osteoclastic bone resorption involves various events including attachment of osteoclasts on bone matrix through the sealing zone to form a resorption compartment, formation of the ruffled membrane where protons and proteinases are secreted into the resorption compartment to degrade bone, and removal of degraded products via transcytosis (3;4).

Tartrate-resistant Acid Phosphatase (TRAP) is an iron-binding protein that is abundantly expressed in mature osteoclasts (5-7). TRAP has been shown to play an important role in bone resorption. In vitro studies demonstrated that a neutralizing antibody against TRAP inhibited bone resorption (8). Confirming the in vitro data, mice lacking TRAP exhibited a defect in endochondral ossification and a mild osteopetrosis (9). Conversely transgenic mice over-expressing TRAP resulted in a decrease in trabecular bone density with a characteristic of a mild osteoporosis (10). Recent studies have suggested that TRAP regulates bone resorption by mediating the degradation of endocytosed matrix products during transcytosis in activated osteoclasts (5;11;12).

TRAP expression is often undetectable in osteoclast precursors but its expression is dramatically up-regulated during osteoclast differentiation (7). As a result, TRAP has been widely used as a marker for osteoclasts (2). Since the discovery of RANKL, it has been established that RANKL plays a key role in TRAP expression during osteoclast differentiation (13;14). However, the molecular mechanism by which RANKL regulates TRAP expression during osteoclast differentiation still remains unknown.

RANKL, identified as a member of the TNF superfamily (13;15-17), is a potent activator of osteoclast differentiation, function and survival (18;19). RANKL exerts its effects by binding to its receptor RANK which was identified as a member of the TNF receptor family (16). Upon
RANKL binding, RANK interacts with various TRAFs to initiate intracellular signaling pathways leading to the activation of two transcription factors: NF-κB and AP-1 (18;20;21). Both NF-κB and AP-1 play an essential role in osteoclast differentiation and function (22;23).

Murine TRAP promoter was previously cloned and characterized (24-26). Importantly, a 1.8-kb mouse promoter region is capable of driving the osteoclast-specific expression of Src gene fused downstream of the TRAP promoter, in transgenic mice (27), indicating that this 1.8-kb promoter contains important regulatory elements required for transcriptional activation in osteoclasts. In our present study, we investigated the molecular mechanism underlying the RANKL-dependent TRAP expression during osteoclast differentiation by using a luciferase reporter construct containing the 1.8-kb TRAP promoter. We identified a 12-bp sequence in the TRAP promoter that is involved in TRAP transcriptional activation in response to RANKL. Interestingly, we also showed that this 12-bp sequence regulates the RANKL-induced TRAP gene transcription by using upstream stimulating factors (USF) 1 and 2, instead of NF-κB and AP-1 that are known transcription factors activated by RANKL. These data not only establish a functional role of USF1 and USF2 in TRAP expression in osteoclasts but also define a new transcriptional mechanism by which RANKL regulates gene transcription.
EXPERIMENTAL PROCEDURES

Chemicals and Reagents - Chemicals were purchased from Sigma (St. Louis, MO) unless indicated otherwise. Synthetic oligonucleotides 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), c-Myc (sc-674X), Max (sc-197X), USF-1 (sc-229X) and USF-2 (sc-862X), were from Santa Cruz Biotechnology, Inc (Santa Cruz, CA)

Construction of a TRAP Promoter-Luciferase Reporter Plasmid and Deletion Mutants - A 1,858-bp mouse TRAP promoter (from –1858 to 1- in relation to ATG) was amplified by PCR using primers derived from published sequences (24-26). The forward primer (5’-cgggatcccccgggtctcccttaactcctgggac-3’) contains a Bam HI site (underlined) at its 5’ end whereas the reverse primer (5’-cgaagctttgaggaagagggagttcagag-3’) has a Hind III site (underlined) at its 5’ end. The PCR was performed using genomic DNA from C3H mice from Harlan Industries (Indianapolis, IN) as a template and high fidelity Pfx polymerase from Invitrogen (Carlsbad, CA). PCR products were digested with Bam HI and Hind III and subcloned into a luciferase reporter plasmid pGL3-basic (Promega, Madison, WI) between Bgl II and Hind III. Cohesive ends resulting from Bam HI and Bgl II digestion are compatible. The amplified TRAP promoter was confirmed by sequencing and the resulting reporter plasmid was named TP(-1858).

9 deletion mutants of TP(-1858), TP(-1239), TP(-1199), TP(-1159), TP(-1119), TP(-1079), TP(-1039), TP(-839), TP(-639) and TP(-439), were prepared using forward primers derived from different 5’ positions (the numbers in parentheses) of the TRAP promoter and the same reverse primer as used above for amplifying the longest construct TP(-1858). All of these forward primers contain a Bam HI site at their 5’ ends. PCR was performed using TP(-1858) as a template and high fidelity Pfx polymerase from Invitrogen. The amplified TRAP promoter fragments were subcloned into pGL3-basic (Promega) and their sequence was confirmed by sequencing.

Cell Cultures and Transient Transfections - RAW 264.7 cells (ATCC, Manassas, VA) were cultured in DMEM containing 10% heat-inactivated FBS in tissue culture plates and passed by
lifting the cells by scraping. Cells were transiently transfected using LipofectAmine Plus transfection reagents from Invitrogen. For transfections, cells were plated in 6-well cell culture plates at the concentration of $8 \times 10^5$ cells/well one day before transfections. For each well, 2 ug reporter plasmid plus 0.05 ug internal-control plasmid phRL-SV40 (Promega) were used. Transfected cells were treated with or without 200ng/ml GST-RANKL for various times after transfection and lysed for luciferase assays using Dul-Luciferase Reporter Assay System from Promega.

**Nuclear Extract Preparation** - Bone marrow macrophages (BMMs) were isolated from long bones of 4-8 week old C3H mice from Harlan Industries as described (28) and cultured in α-MEM containing 10% heat-inactivated FBS in the presence of 10ng/ml recombinant M-CSF (R&D Systems, Minneapolis, MN). RAW 164.7 cells were cultured as described above in tissue culture dishes. Cells were treated with or without RANKL treatment for indicated times. Upon confluence (about 1X$10^7$ cells), cells were washed with cold PBS 3 times. Cells were then scraped off the dishes, spun down, resuspended in 1.5 ml of cold PBS, and transferred to 2-ml microcentrifuge tubes. Cells were pelleted in a microcentrifuge for 30 seconds, media were removed, and the cells were resuspended in 500 ul of Hypotonic Lysis Buffer (10 mM Hepes-KOH, pH 7.9, 10mM KCl, 1.5 mM MgCl$_2$, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride; DTT and phenylmethylsulfonyl fluoride were added freshly). Cells were lysed for 15 min on ice, at which time 32 ul of 10% Nonidet P-40 was added to the suspension, followed by vortexing the tube for 15 seconds and incubating on ice for 10 min. Nuclei were spun down and resuspended in 100 ul of Nuclear Extraction Buffer (20 mM Hepes-KOH, pH 7.9, 420 mM NaCl, 1.2 mM MgCl$_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM BAESF, 5ug/ml pepstatin, and 5 ug/ml leupeptin. DTT, phenylmethylsulfonyl fluoride, BAESF, pepstatin, and leupeptin were added freshly to the buffer). The nuclei were incubated with the extraction buffer on ice for 20 min and spun down in a microcentrifuge. The supernatant (nuclear extract) was aliquoted, quickly frozen in dry ice/ethanol bath, and stored at –
80°C. Protein concentration of nuclear extracts was determined using Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

**Electrophoretic Mobility Shift Assays (EMSAs)** - Oligonucleotides (oligos) used for EMSAs were end-labeled with $^{32}$P by T4 polynucleotide kinase (Invitrogen). 2-5X10$^4$ cpm probe was incubated with 3 ug of nuclear extracts in a 20-ul volume of binding reaction (10mM Tris-Cl, pH7.5, 100mM NaCl, 10% glycerol, 50ng/ml poly(dI/dC) on ice for 20 min. In competition experiments, a 50X and/or 100X excess amount of unlabeled competitors was premixed with labeled probe before being added to the binding mixture. The binding reaction was then allowed to proceed for 20 min on ice. In supershift experiments, probe was incubated with 3 ug/ml nuclear extracts in a 20-ul volume of binding reaction for 20 min on ice, at which time 4 ug control IgG or 4 ug specific antibodies were added, followed by incubation on ice for an additional 30 min. All binding mixtures were separated, using 0.5XTBE buffer as the running buffer, at 4°C and 100 V for 3.5 h by 4-20% gradient TBE gels (Invitrogen) in Novex Xcell II minicell electrophoresis system. The gels were transferred to 3M blotting paper, dried and exposed to film.

**In vitro translations** - In vitro translated USF1 and USF2 were prepared by using PROTEINscript™ II Linked Transcription/Translation Kit (Ambion, Austin, TX) and expression plasmids for USF1 (psvUSF1) and USF2 (psvUSF2) described in (29). These expression plasmids were constructed using vector pSG5 (Stratagene, La Jolla, CA) that contains T7 promoter suitable for in vitro translations. Thus, PROTEINscript II T7 was used for in vitro translation assays with these expression vectors. Briefly, 0.5 ug of each plasmid (pSG5, psvUSF1 or psvUSF2) was used to set up transcription reactions (10 ul reaction volume for each experiment) following the protocol provided by the manufacturer. Subsequently, 2 ul of the transcription reaction was used for the translation reaction following the protocol provided by the manufacturer (50 ul reaction volume for each experiment). 5 ul from each translation reaction was then used to perform EMSA as described above.
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). Oligos used to mutate the USF-binding site are 5’-GCTACAGCCAGCCACGgaGTGTGTGCCTTCTGG-3’ and 5’-CCAGAAGGCACACACtcCGTGGCTGGCTGTAGC-3’. These oligos were purified by polyacryamide gel electrophoresis. PCRs were performed in a 50-ul volume with Pfu polymerase, 10 ng of DNA template TP(-1858), and 125 ng of each oligo using the following conditions: 95°C for 30 s, 1 cycle; 95°C for 30 s, 55°C for 1 min and 68°C for 12 min, 18 cycles; and 4°C park. The PCR products were treated with Dpn I (10 units) for 60 min at 37°C. XL1-blue supercompetent cells were transformed with the Dpn I-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 correctness of introduced mutations.

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

A 1,858-bp mouse TRAP promoter confers responsiveness to RANKL – Osteoclasts were previously prepared in vitro by co-culturing BMMs (osteoclast precursors) with osteoblasts or stromal cells in the presence of osteotropic factors such as 1α,25(OH)2 vitamin D3 and dexamethasone (30-32). The discovery of RANKL as a critical osteoclastogenic factor has revolutionized the method of in vitro osteoclast preparation. Osteoclasts can now be generated simply by treating BMMs with M-CSF and RANKL (13;15). More importantly, the previous studies showed that RANKL significantly increases TRAP mRNA levels both in primary BMMs (13) and in RAW264.7 cells (14), suggesting that RANKL may upregulate TRAP expression by activating the transcription of TRAP gene. To investigate this possibility, we amplified a 1,858-bp mouse TRAP promoter (–1858 to –1, relative to the translation start site ATG) by PCR based on published sequence (24-26). The nucleotide sequence of our clone is compared with those published and differences exist among these three clones (Fig. 1A). To confirm that our sequence is not altered by possible mutations associated with PCR-based amplifications, we sequenced and compared two clones from two independent PCR amplifications. Thus, the differences between our clone and two published ones likely result from variations in mouse strains and/or individual animals.

The amplified TRAP promoter was subcloned into pGL3-basic in sense orientation to luciferase gene to generate a reporter construct TP(-1858). Since primary BMMs are extremely difficult to transfect, we used RAW264.7 cells for our transfection assays. TP(-1858) was transiently transfected into RAW264.7 cells, and the transfection result shows that RANKL activated the TRAP promoter in a time-dependent manner with a maximal induction at day 4 (about 2.5-fold) (Fig. 1B), thus indicating that the 1,858-bp TRAP promoter contains a sequence(s) mediating RANKL-induced activation of TRAP gene transcription. In addition, since the data show that a 4-day RANKL treatment gave rise to the highest TRAP promoter activation, all of our following transfection studies will be performed with a 4-day RANKL treatment.
Localization of a 40-bp TRAP promoter region (-1239 to –1199) that binds RANKL-induced nuclear proteins - To characterize the sequence(s) involved in the RANKL-induced TRAP transcription, we generated 5 deletion mutants of the TRAP promoter: TP(-1239), TP(-1039), TP(-839), TP(-639) and TP(-439) (Fig. 2A). These mutants contain RANKL promoter regions starting from different 5’ positions (the numbers in parentheses) and ending at the same 3’ site (-1). We then used these deletion mutants to perform transfection studies (Fig. 2B). While our longest construct TP(-1858) gave rise to about 2.5 fold increase in TRAP promoter activity in response to RANKL, deletion of a 619-bp region (-1858 to -1239) only resulted in about 1.6 fold induction (Fig. 2B), indicating that this 619-bp region plays a role in RANKL-dependent TRAP gene transcription. Moreover, deletion of another 200-bp region (-1239 to -1039) totally abolished RANKL responsiveness (Fig. 2B), revealing that this 200-bp region is also critical for RANKL-dependent TRAP gene transcription. Taken together, these data show that two TRAP promoter regions are involved in RANKL-dependent TRAP transcription.

In this study, we chose to focus on characterizing the cis-element(s) in the 200-bp region (-1239 and –1039). To this end, we first determined whether the 200-bp region binds any nuclear proteins in response to RANKL. We synthesized 5 overlapping oligos spanning the entire 200-bp TRAP promoter region (Fig. 2C). We labeled these oligos and used them as probes to perform EMSA with nuclear extracts from untreated and RANKL-treated RAW264.7 cells (Fig. 2D). Oligo I gave rise to a major band (band A) in EMSA with untreated RAW264.7 cells (lane 2). This band was significantly enhanced when RANKL-treated RAW264.7 nuclear extracts were used (lane 3), suggesting that nuclear proteins corresponding to band A may be implicated in RANKL-induced TRAP transcription. In addition to band A, a minor band with a higher mobility (band B) was also observed (lanes 2 and 3). However, in our subsequent EMSAs, band B appears to be very unstable. Thus, we only focused on band A in the present study. Interestingly, oligo IV also binds nuclear proteins induced by RANKL in our EMSA (lanes 11 and 12). These data
suggest that oligo I and oligo IV may contain cis-elements regulating RANKL-induced TRAP transcription.

The 40-bp TRAP promoter region (-1239 to -1199) mediates RANKL-induced TRAP transcription via transcription factors other than NF-κB and AP-1 - Next, we prepared 4 additional deletion mutants in which a 40-bp promoter region was deleted in a progressive fashion starting from -1239 (Fig. 3A). Consistent with the EMSA above (Fig. 2D), our transfection studies with these mutants indicate that the 40-bp region from -1239 to -1199 is critical for RANKL-induced TRAP promoter activation (Fig. 3B), confirming that oligo I contains a cis-element(s) regulating RANKL-induced TRAP transcription.

The previous studies have well established that RANKL activates two transcription factors NF-κB and AP-1 (18;20;21), which play a pivotal role in osteoclast differentiation (22;23). However, our computer analysis found no consensus sequences for NF-κB or AP-1 in this 40-bp region, suggesting that the nuclear proteins binding to this 40-bp sequence are not NF-κB or AP1. To experimentally confirm this, we performed a competition assay using excess cold oligo I and oligos containing NF-κB/AP-1 consensus sequences as competitors (Fig. 4A). While excess cold oligo I were able to compete for the nuclear protein binding, both NF-κB and AP-1 oligos failed to do so, further suggesting that this 40-bp does not bind NF-κB or AP-1. Subsequently, our supershift assays with antibodies against p50, P65, c-fos and c-jun confirmed that the 40-bp sequence binds transcription factors other than NF-κB and AP-1 (Fig. 4B).

Identification of a 12-bp sequence AGCCACGTGGTG that specifically binds RANKL-induced USF1 and USF2 in oligo I - Upon the exclusion of NF-κB and AP-1 as the nuclear proteins binding to oligo I, we proceeded to elucidate the identity of the nuclear proteins. Our computer analysis revealed that this 40-bp region contains putative binding sequences for many
transcription factors, including ELP/SF1/FTZ (33), USF (34;35), PPAR (36), CP1/2 (37), PHO4 (38;39) and c-Myc (40). Since this information is not specific enough, we decided to identify the specific nuclear protein-binding sequence as our first step in elucidating the nuclear proteins. In doing so, we performed a series of competition assays using shortened oligos derived from oligo I as competitors (Fig. 5A). As shown in Fig. 5B, while SOI 1 competed efficiently for the nuclear protein binding (lane 2), SOIs 2-5 failed to do so (lanes 3-6), revealing the 5’ end of the specific nuclear protein-binding sequence (Fig. 5A). Similarly, the experiment in Fig. 5C showed that SOIs 6-11 could compete efficiently (lanes 2-7) but not SOI 12 (lane 8), elucidating the 3’ end of the sequence (Fig. 5A). Together, these data identified a 12-bp sequence within the 40-bp region that binds specifically the nuclear proteins induced by RANKL (Fig. 5A).

The 12-bp sequence contains a sequence CACGTG, which is recognized as a core sequence present in binding sites for both the Myc family members (40) and USF proteins (34;35). This suggests that the nuclear proteins binding to the 12-bp sequence might be members of the Myc family or USF proteins. As a result, we performed supershift assays with antibodies against c-Myc, c-Max, USF1 and USF2 (Fig. 6A). While antibodies against c-Myc and c-Max had no effect on band A (lanes 3 and 4), those against USF1 and USF2 supershifted the band (lanes 5 and 6), confirming that the nuclear proteins binding to the 12-bp sequence are USF1 and USF2. Moreover, USF1 antibody only partially supershifted band A, resulting in a weak band (lane 5). In contrast, USF2 antibody completely supershifted the band (lane 6). These data further indicate that this 12-bp sequence is able to bind USF proteins from RAW264.7 cells as either USF1-USF2 heterodimers or USF2 homodimers.

At this point we identified a 12-bp sequence in TRAP promoter that binds USF1 and USF2 from RAW264.7. Since TRAP gene expression is also activated by RANKL in primary BMMs, we examined whether this sequence also binds RANKL-induced USF1 and USF2 from primary BMMs. As shown in Fig. 6B, oligo I binds nuclear proteins from untreated primary BMMs, resulting in two bands (A and B, lane 1). Band A is significantly enhanced when nuclear extracts...
from RANKL-treated BMMs were used (lane 2). Consistently, the pattern of banding in the EMSA (Fig. 6B) is very similar to that seen in the EMSA with nuclear extracts from RAW264.7 cells (Fig. 2D, lanes 2 and 3). Importantly, supershift assays indicate that the nuclear proteins binding to oligo I (band A) from primary BMMs treated with RANKL are also USF1 and USF2 (Fig. 6C). These results further support that the 12-bp sequence in TRAP promoter may play a functional role in RANKL-dependent TRAP transcription by utilizing USF1 and USF2.

As additional evidence supporting that this 12-bp sequence binds USF1 and USF2, we performed EMSA using in vitro translated USF1 and USF2, which were prepared by using expression vectors psvUSF1 (for USF1) and psvUSF2 (for USF2) described in (29). These expression plasmids were constructed using vector pSG5 (29). As shown in Fig. 7, oligo I does not bind any proteins from in vitro translation reaction with control vector pSG5 in EMSA (lane 1). In contrast, oligo I binds proteins from in vitro translation reaction with psvUSF1 (lane 2) or psvUSF2 (lane 3). As expected, oligo I binds both USF1 and USF2 when both in vitro translated USF1 and USF2 were used in the EMSA (lane 4). Notably, 5 ul of the in vitro translation reaction with psvUSF2 gave rise to much weaker band (lane 3) than that obtained with the same volume of the translation reaction with psvUSF1 (lane 2). This assay was independently repeated twice and the same result was obtained. This discrepancy may result from a difference in the affinities of these in vitro translated USF proteins for the 12-bp sequence or from the low translation efficiency associated with psvUSF2. Nevertheless, these data strongly supports that the 12-bp sequence is a USF-binding site.

The 12-bp USF binding sequence is functionally involved in the RANKL-induced TRAP transcription - Finally we determined whether the 12-bp USF-binding sequence is functionally involved in the RANKL-dependent TRAP transcription. To this end, we need to identify a mutation in the USF-binding site that is capable of blocking the USF binding. We synthesized a mutant oligo I (mOligo I) in which TG, in the core binding CACGTG, were converted to GA
(Fig. 8A). Competition assays show that mOligo I failed to compete for the USF binding (lane 3, Fig. 8B), indicating that the chosen mutation is sufficient to eliminate the binding capacity of the sequence. To exclude the possibility that the chosen mutation, although it abolish USF recognition, render the sequence capable of associating with other nuclear proteins, mOligo I was used as probe to perform an EMSA (Fig. 8C). The data confirm that mOligo I did not bind any additional nuclear proteins.

We introduced the same mutation in our longest reporter construct TP(-1858), resulting in a reporter construct named mTP(-1858) (Fig. 8D). In transfected RAW264.7 cells, while TP(-1858) resulted in 2.5 fold induction in response to RANKL, mTP(-1858) gave rise to only 1.8 fold induction (p<0.01) (Fig. 8E), indicating that the 12-bp USF binding sequence is functionally involved in RANKL-induced transcriptional activation of TRAP gene.
DISCUSSION

TRAP expression is dramatically up-regulated during osteoclast differentiation (7). In mature osteoclasts, TRAP plays an important role in osteoclastic bone resorption (8-10). As a result, elucidation of the regulatory mechanism controlling TRAP expression in osteoclasts has long been an active part of bone biology research. Prior to the discovery of the RANKL/RANK system in the late 1990’s, osteoclasts were generated in vitro virtually by co-culturing primary BMMs with osteoclasts/stromal cells (30-32), which were believed to provide essential osteoclastogenic factors (30;41). However, the identities of these factors were unknown. Consequently, the identity of the molecule(s) regulating TRAP expression in osteoclasts was also unclear. Since the unraveling of the RANKL/RANK system, it has now been established that osteoblasts/stromal cells support osteoclast differentiation in the co-culture system primarily by producing two critical factors: M-CSF and RANKL (13;15), which play an essential role in osteoclast differentiation (2). Moreover, it has also become clear that RANKL is an essential and potent factor involved in the up-regulation of TRAP expression during osteoclast differentiation (13;14). These findings prompted us to investigate the molecular mechanism by which RANKL regulates TRAP expression during osteoclast differentiation.

Our studies took advantage of a macrophage-like cell line RAW264.7, which is not only transfectable but it is also capable of differentiating into osteoclasts in response to RANKL (14). Significantly, our initial transfection studies showed that two distinct TRAP promoter regions are important for TRAP transcriptional activation in response to RANKL, suggesting that multiple transcriptional events are involved in RANKL-induced TRAP gene activation. A detailed characterization of one such region in this paper revealed that a 12-bp sequence AGCCACGTGGTG is involved in enhancing TRAP gene transcription in response to RANKL. More significantly, we further showed that this 12-bp sequence does so by binding USF1 and USF2.
Transcription factors USF1 and USF2 were originally identified by their ability to bind to the adenovirus major late promoter (42). Structurally, USF1 and USF2 are related to the Myc family of transcription factors, which are characterized by the presence of a C-terminal basic helix-loop-helix-leucine zipper (bHLH-Zip) domain (33;43). USF1, USF2 and the Myc family members were shown to recognize DNA sequences containing a core sequence CACGTG (29). While the RANKL-responsive sequence (AGCCACGTGGTG) identified in TRAP promoter contains such a core sequence (underlined), our data showed that it binds USF1 and USF2 but not c-Myc or Max in response to RANKL (Fig. 6A and Fig. 6C). This indicates that this 12-bp RANKL-responsive sequence regulates RANKL-induced TRAP transcription exclusively by utilizing USF1 and USF2. Notably, it was recently demonstrated that RANKL enhances significantly c-Myc expression in RAW264.7 cells during osteoclast differentiation (14). However, the precise role of c-Myc in TRAP transcription is controversial. Over-expression of a dominant negative c-Myc mutant in RAW264.7 cells resulted in a reduction in the levels of TRAP transcripts (14), supporting a positive role of c-Myc in TRAP expression. In contrast, c-Myc was recently shown to have negative effect on TRAP transcription when overexpressed in a murine macrophage cell line P388D1 (44).

It has been previously shown that various other transcription factors are implicated in regulating TRAP transcription in osteoclasts. Particularly, the Microphthalmia transcription factor MITF, a critical regulator of osteoclast function (45), plays an important role in controlling TRAP transcription (46). Similar to USF1 and USF2, MITF is also characterized as a bHLH-Zip protein, and it regulates TRAP transcription by binding to a conserved sequence GGGTCATGTGAGC (located at –568 to –556 in mouse TRAP promoter) containing a M-Box (underlined)(46). In agreement with its role in TRAP transcriptional activation, MITF was shown to be a target of RANKL action in osteoclasts (47). RANKL activates MITF by phosphorylating MITF at Ser\textsuperscript{307} via the p38 MAPK pathway (47). Furthermore, MITF regulates TRAP transcription in collaboration with PU.1 (48), TFE-3 and TFE-C (49). TFE-3 and TFE-C are also characterized as
bHLH-Zip proteins and closely related to MITF (49). Our data not only identified USF1 and USF2 as additional members of the bHLH-Zip superfamily that play a critical role in controlling TRAP transcription, but also raised questions on potential collaborations of USF1 and USF2 with the previously characterized bHLH-Zip transcription factors in regulating TRAP transcription in osteoclasts.

Although NF-κB and AP-1 are two well-characterized transcription factors activated by RANKL in osteoclast differentiation, our present work reveals that RANKL regulates TRAP gene transcription by activating USF1 and USF2 (e.g.: enhancing binding of USF1 and USF2 to the 12-bp RANKL-responsive sequence) in osteoclasts. How does RANKL activate USF proteins? One possibility is that RANKL increases the USF gene expression, and the enhanced USF binding directly results from the increase in amounts of USF proteins available. Alternatively, RANKL has no effect on the USF gene expression. Instead, RANKL activates USF proteins by phosphorylating them and phosphorylated proteins have higher affinity for the binding site. The latter represents a more reasonable hypothesis because: 1) RANKL is able to activate the p38 MAPK pathway (50-52) and 2) the p38 MAPK pathway was shown to play a role in phosphorylating USF1 in mediating UV-induced Tyrosinase expression in a mouse melanocyte cell line (53). Nonetheless, the precise mechanism of the RANKL-mediated USF activation is currently under investigation. Elucidation of the signaling pathway involved in RANKL-mediated USF activation will provide more insights into the molecular mechanism by which RANKL regulates osteoclast differentiation.

Our initial transfection data showed that two distinct TRAP promoter regions are involved in regulation of TRAP transcription in response to RANKL (Figs. 2A and 2B). Consistently, mutation of the USF-binding site in the region at -1239 to -1039 only partially blocked the RANKL-induced TRAP transcriptional activation (Fig. 8E), further supporting that the other region at -1858 to -1239 also contributes to the RANKL-induced TRAP transcriptional activation. To fully elucidate the molecular mechanism underlying RANKL-dependent TRAP
gene activation, the cis-elements in the region at –1858 to -1239 need to be characterized. Furthermore, oligo IV binds a nuclear protein(s) (band C, lane 12, Fig. 2C) in response to RANKL. Given that mutant constructs TP(-1199) and TP(-1159) failed to confer RANKL responsiveness, the nuclear protein(s) binding to oligo IV is not sufficient to activate TRAP promoter in response to RANKL. However, our data by no means exclude the possibility that the nuclear protein(s) are necessary for RANKL-induced TRAP gene activation. Thus, investigation of the potential role of the nuclear protein(s) in RANKL-dependent TRAP transcription represents is warranted.

In conclusions, our data presented here demonstrate that USF1 and USF2 play an important role in RANKL-dependent TRAP transcription. This finding not only defines a role of USF proteins in TRAP expression in osteoclasts, but more importantly it also raises many important questions. First, whether and how USF proteins collaborate with other transcription factors in regulating TRAP transcription is unknown. Furthermore, the precise signaling pathway by which RANKL activates USF transcription factors remains to be elucidated. Future studies aimed at addressing these questions will provide more insights into molecular mechanisms governing osteoclast differentiation and function.
ACKNOWLEDGMENT

This work is partially supported by a National Institutes of Health grant AR 47830 (X.F.), NIH RCC grant: UAB Core Center for Musculoskeletal Disorders (P30AR46031), a National Osteoporosis Foundation grant (X.F.), a pilot grant from Center for Aging at the University of Alabama at Birmingham (UAB) (X.F.) and an ACS IRG from UAB Comprehensive Cancer Center (X.F.).
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FOOTNOTES

1. The abbreviations used are: TRAP, tartrate-resistant acid phosphatase; USF, upstream stimulatory factor; BMM, bone marrow macrophages; RANK, receptor activator of NF-κB; RANKL, RANK ligand; M-CSF, monocyte/macrophage colony stimulating factor; TNF, tumor necrosis factor; TRAF, TNF receptor associated factor; EMSA, electrophoretic mobility shift Assay; bHLH-Zip, basic helix-loop-helix-leucine zipper (bHLH-Zip) domain; MITF, Microphthalmia transcription factor.

2. The nucleotide sequence for the cloned TRAP promoter has been deposited in the GenBank database under GenBank Accession Number AY187311.
FIGURE LEGENDS

Fig. 1. A 1,858-bp mouse TRAP promoter confers responsiveness to RANKL. A, a comparison of our TRAP promoter sequence with those previously published (24-26). Our TRAP promoter was amplified by PCR using the forward and reverse primers as shown in the figure, derived from the two published sequences. The amplified clone contains a genomic region from -1,858 to -1, relative to the translation start site ATG shown in bold. Here we mainly show regions containing variations among our clone and the published sequences. All conserved sequences are in capital letters. Lower case letters highlight the differences. The 12-bp USF-binding sequence mediating RANKL-induced TRAP transcription is underlined. Ours: the clone we amplified by PCR (GenBank AY187311). Pub1: the clone reported by Reddy and co-workers (GenBank M85212) (24). Pub2: the clone reported by Cassady and co-workers (GenBank M99054) (25). B, the 1,858-bp TRAP promoter mediates transcriptional activation in response to RANKL. RAW264.7 cells were transiently cotransfected with TP(-1858) and internal-control plasmid phRL-SV40. Transfected cells were treated with RANKL (200ng/ml) or PBS as control for 1, 2, 3, or 4 days. Firefly luciferase activity of TP(-1858) was normalized to Renilla luciferase activity of internal control phRL-SV40. The experiment was repeated twice and one result is shown. Each bar is the mean of three replicates ± S.D.

Fig. 2. Localization of a 40-bp TRAP promoter region (-1239 to –1199) that binds RANKL-induced nuclear proteins. A, construction of 5 deletion mutants of TRAP promoter: TP(-1239), TP(-1039), TP(-839), TP(-639) and TP(-439). These mutants were constructed by PCR as described in Experimental Procedures. B, transfection assays. RAW264.7 cells were cotransfected with the deletion mutants shown in Fig. 2A and internal-control plasmid phRL-SV40. Transfected cells were treated with RANKL (200ng/ml) or PBS as control for 4 days. Firefly luciferase activities of these mutants were normalized to Renilla luciferase activity of
internal control phRL-SV40. The experiment was repeated four times and a representative result is shown. Each bar is the mean of three replicates ± S.D. C, diagram of 5 overlapping oligos (I, II, III, IV and V) spanning the 200-bp TRAP promoter region (-1239 to -1039). D, EMSA with oligos I, II, III, IV and V. Oligos I-V shown in Fig. 2C were labeled and used as probes to perform EMSA with nuclear extract (N.E.) from untreated RAW264.7 cells (U) or RAW264.7 cells treated with RANKL (200ng/ml) for 4 days (T). C: BSA was added as control. Three RANKL-induced bands are indicated by arrows A, B and C.

Fig. 3. The 40-bp TRAP promoter region corresponding to oligo I mediates RANKL-induced TRAP transcription. A, construction of deletion mutants, TP(-1239), TP(-1199), TP(-1159) and TP(-1119). These mutants were constructed by PCR as described in Experimental Procedures. B, transfection studies with the deletion mutants in Fig. 3A. RAW264.7 cells were transfected with the deletion mutants as in Fig. 2B. The experiment was repeated four times and a representative result is shown. Each bar is the mean of three replicates ± S.D.

Fig. 4. Oligo I binds transcription factors other than NF-κB and AP-1. A, competition assays. A competition assay was performed using labeled oligo I as probe and nuclear extracts (N.E.) from RAW264.7 cells treated with RANKL (200ng/ml) for 4 days: RAW(RANKL). Excess cold oligo I (lanes 2 and 3), NF-κB oligos (lanes 4 and 5) and AP-1 oligos (lanes 6 and 7) were added as competitors (Comp). As control, no competitor was added in lane 1 (None). NF-κB oligos and AP-1 oligos are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). B, supershift assays. A supershift assay was performed using labeled oligo I as probe and nuclear extracts (N.E.) from RAW264.7 cells treated with RANKL (200ng/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.
Fig. 5. Identification of a 12-bp sequence binding the nuclear proteins in oligo I using competition assays. A, diagram of shortened oligos I used for competition assays. Shortened oligo I (SOI) 1-5 have the same 5’-ends but different 3’-ends. In contrast, SOI 6-12 have different 5’-ends but the same 3’-ends. The identified 12-bp sequence is shown in bold. Two broken lines highlight the 5’- and 3’- ends of the sequence. B, localization of the 3’-end of the sequence binding the RANKL-induced nuclear proteins. A competition assay was performed using labeled oligo I as probe and SOI 1-5 as competitors (Comp). Nuclear extracts (N.E.) used for this assays were from RAW264.7 cells treated with RANKL (200ng/ml) for 4 days: RAW(RANKL). C, localization of the 5’-end of the sequence binding the RANKL-induced nuclear proteins. The experiment was performed as described in Fig. 5B except that SOI 6-12, instead of SOI 1-5, were used.

Fig. 6. Elucidation of the identity of nuclear proteins binding to oligo I using supershift assays. A, Oligo I binds USF1 and USF 2 induced by RANKL from RAW264.7. The assay was performed using labeled oligo I as probe and nuclear extracts (N.E.) from RAW264.7 cells treated with RANKL (200ng/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; c-Myc in lane3; c-Max in lane 4; USF1 in lane 5 and USF2 in lane 6. SS: supershift. B, oligo I also binds RANKL-induced nuclear proteins from primary BMMs. EMSA was performed using labeled oligo I as probe and nuclear extracts (N.E.) from primary BMMs. U: BMMs treated with M-CSF alone (10ng/ml) for 4 days. T: BMMs treated with M-CSF (10 ng/ml) and RANKL (200 ng/ml) for 4 days. Similar to EMSA with nuclear extracts from RAW264.7 cells, in this EMSA oligo I gave rise to a major band (indicated by arrow A), which was greatly enhanced in response to RANKL treatment. C, RANKL-induced nuclear proteins are USF1 and USF2. Supershift assay was performed using labeled oligo I as probe and nuclear extracts (N.E.) from primary BMMs treated
with M-CSF (10ng/ml) and RANKL (200ng/ml) for 4 days: BMM(RANKL). No antibody was added in lane 1 (None). Different antibodies were added in lanes 2-6: IgG in lane 2 as control; c-Myc in lane 3; c-Max in lane 4; USF1 in lane 5 and USF2 in lane 6. SS: supershift.

**Fig. 7. Oligo I binds in vitro translated USF1 and USF2.** USF1 and USF2 were in vitro translated as described in Experimental Procedures. EMSA was performed using labeled oligo I as probe and 5 ul of the translation reaction with empty vector pSG5 as control (lane 1), 5 ul of the translation reaction with psvUSF1 (lane 2), 5 ul of the translation reaction with psvUSF2 (lane 3), or 5 ul of the translation reaction with psvUSF1 plus 5 ul of the translation reaction with psvUSF2.

**Fig. 8. Mutation of the USF-binding site results in a reduction in RANKL-induced TRAP promoter activity.** A, a mutant oligo I (mOligo I) contains two point mutations (conversion of GT to ga, highlighted here by lower case letters) in the USF-binding site. Wild-type oligo I is also shown for comparison. The 12-bp USF binding site is in bold. The core sequence CACGTG is shown in boxes. B, mOligo I fails to compete for USF binding in a competition assay. The competition assay was performed using labeled oligo I as probe and 100x excess cold oligo I (lane 2) or mOligo (lane 3) as competitors (Comp). Nuclear extracts (N.E.) used for this assay were from RAW264.7 cells treated with RANKL (200ng/ml) for 4 days: RAW(RANKL). C, mOligo I does not bind USF proteins in EMSA. EMSA was performed using labeled oligo I (lane 1) and mOligo I (lane 2) as probe and nuclear extracts (N.E.) from RAW264.7 cells treated with RANKL (200ng/ml) for 4 days: RAW(RANKL). D, diagram of mTP(-1858). mTP(-1858) was generated by introducing the two point mutations shown in Fig. 8A. E, transfection assays with mTP(-1858). RAW264.7 cells were transiently cotransfected with TP(-1858) as control or mTP(-1858) as well as internal-control plasmid phRL-SV40. Transfected cells were treated with RANKL (200ng/ml) or PBS as controls for 4 days. Firefly luciferase activities of TP(-1858) and
mTP(-1858) was normalized to *Renilla* luciferase activity of internal control phRL-SV40. This experiment was independently repeated three times and each experiment had three replicates. A fold induction was calculated for each experiment using the averages of its three replicates. Each bar is the mean of three fold induction values from these independent experiments ± S.D. (P<0.01).
A

Pub1 (−1846) CCCGGGTCTCCCTTAACCTGCTGCGC (−1798) 
Pub2 (−1858) CCCGGGTCTCCCTTAACCTGCTGCGC (−1810) 
Ours (−1858) CCCGGGTCTCCCTTAACCTGCTGCGC (−1809) 

Bam HI Forward Primer

Pub1 GgacAgGctgGcCC (−1694) CA. GC (−1580) G..aG (−1556) 
Pub2 GgcAgAagCtGgcCC (−1706) CAgGC (−1591) GaagG (−1565) 
Ours GgcAgGctgGcCC (−1705) CAgGC (−1590) GgagG (−1564) 

Pub1 GGGgCCAC (−1533) TcGCCT (−1511) GTG. AGTCG (−1447) 
Pub2 GGGgCCAC (−1542) T. GCCT (−1521) GTG. AGTCG (−1457) 
Ours GGGgCCAC (−1541) TcGCCT (−1519) GTGaAGTCG (−1454) 

Pub1 G. ..CTG (−1238) ACAGGCCAGGCACTGTTGCTGCTCCTTC (−1186) 
Pub2 GctgaACTG (−1244) ACAGGCCAGGCACTGTTGCTGCTCCTTC (−1192) 
Ours GctgaACTG (−1241) ACAGGCCAGGCACTGTTGCTGCTCCTTC (−1189) 

USF binding site

Pub1 C. AA (−862) ACCACCcagAAAAGaTG (−776) T..cagCC (−728) 
Pub2 C. AA (−868) ACCACCcagAAAAGaTG (−783) TccagCC (−733) 
Ours CaAA (−864) ACCACCcagAAAAGaTG (−778) T..cagCC (−730) 

Pub1 CctGGG.CTG (−650) G. CTT (−637) G. CC (−600) A.TTT (−583) 
Pub2 CtgGGcCTG (−654) GcCTT (−640) GcCC (−602) ATTTT (−584) 
Ours CctGGG.CTG (−652) G. CTT (−639) G. CC (−601) A.TTT (−584) 

Pub1 ATGA.CC (−550) CCAGAGACTCTGAACCTCCCTCTTCCTTCACAG (−1) ATG 
Pub2 ATGAcCC (−550) CCAGAGACTCTGAACCTCCCTCTTCCTTCACAG (−1) ATG 
Ours ATGAcCC (−550) CCAGAGACTCTGAACCTCCCTCTTCCTTCACAG (−1) ATG

Reverse Primer Hind III

B

![Graph showing relative luciferase activity over time](http://www.jbc.org)

**Fig. 1**
### A

| Comp | None | Oligo I | NF-κB | AP-1 |
|------|------|---------|--------|-------|
|      | 50x  | 100x    | 50x    | 100x  |

![Image A](image_a.png)

### B

| Antibody | None | IgG | p50 | p65 | c-fos | c-Jun |
|----------|------|-----|-----|-----|-------|-------|

![Image B](image_b.png)

**Fig. 4**
| Probe       | Oligo I |
|-------------|---------|
| psvUSF1     | -       | +       | -       | +       |
| psvUSF2     | -       | -       | +       | +       |

Fig. 7
Involvement of upstream stimulatory factors 1 and 2 in RANKL-induced transcription of tartrate-resistant acid phosphatase (TRAP) gene during osteoclast differentiation
Yi Liu, Zhenqi Shi, Alexandra Silveira, Jianzhong Liu, Michele Sawadogo, Hongmei Yang and Xu Feng

J. Biol. Chem. published online March 27, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212093200

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