Published in final edited form as:

Biochem Biophys Res Commun. 2007 August 10; 359(4): 1044–1049. doi:10.1016/j.bbrc.2007.06.017.

TRAF6 Ubiquitin Ligase is Essential for RANKL Signaling and Osteoclast Differentiation

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Abstract

Tumor necrosis factor receptor-associated factor 6 (TRAF6), the crucial adaptor molecule of receptor activator of NF- κ B (RANK), plays an essential role in governing the formation of multi-nucleated osteoclasts. TRAF6 is a RING-dependent ubiquitin (Ub) ligase that in conjunction with Ubc13/Uev1A catalyzes its own auto-ubiquitination via Lys63-linked poly-Ub chains. While the receptor-adaptor function of TRAF6 in RANK signaling is well understood, the significance of its Ub ligase activity in this process remains largely unknown. In this study, we show that retroviral expression of TRAF6, but not a RING mutant of TRAF6 was able to rescue TRAF6-deficient monocytes for the activation of IKK and osteoclast differentiation by RANKL. Furthermore, a catalytically inactive Ubc13 or stable knockdown of Ubc13 significantly prevents RANK-mediated TRAF6 ubiquitination and NF- κ B and JNK activation. These data establish a signaling cascade in which regulated Lys63-linked TRAF6 auto-ubiquitination is the critical upstream mediator of osteoclast differentiation.

Keywords

TRAF6; RANK; RANKL; NF- κ B; ubiquitin; RING E3 ubiquitin ligase; osteoclasts

Introduction

Bone remodeling is a dynamic and continuing process of destroying old bone by the resorption activity of osteoclasts and creating new bone by the matrix synthesis activity of osteoblasts. Osteoclasts are fully differentiated, multi-nucleated cells originating from the hematopoietic monocyte-macrophage lineage. RANKL, a member of the tumor necrosis factor (TNF) superfamily, and its receptor RANK are essential regulators of osteoclast maturation and activation [1–4].

Like most members of the TNF receptor superfamily, the cytoplasmic domain of RANK interacts with adaptor proteins of the TRAF family, which participate in activation of the transcription factor NF- κ B and the MAP kinase pathways [5–7]. While TRAF1-3 and 5 bind to the C-terminal tail of RANK, TRAF6 binds to a unique motif located within the membrane proximal region of RANK [8], which was subsequently confirmed by crystallographic analysis [9]. Among all the TRAF knockout studies, only TRAF6-deficient mice displayed bone abnormalities in that they developed osteopetrosis due to a defect in osteoclastogenesis [10–12]. RANKL signaling is disrupted in cells derived from these mice revealing a major role of

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TRAF6 in RANK signaling. Collectively, these data support the indispensable role of TRAF6 in RANK signaling and in terminal differentiation of osteoclast progenitors. However, the molecular mechanism by which TRAF6 exerts its biological activity in RANK signaling is not well understood.

The N-terminal RING domain of TRAF6 belongs to a growing family of Ub ligases, also known as E3s [13]. We have previously demonstrated that the RING domain of TRAF6 and the dimeric E2 enzyme Ubc13/Uev1A are critical for Lys63-linked TRAF6 auto-ubiquitination and the ability of TRAF6 to activate IKK leading to the activation of NF- κ B [14]. Furthermore, we identified a single Lys residue (Lys-124) in TRAF6 as a critical Ub acceptor site and mutation of this site prevented TRAF6-mediated NEMO ubiquitination, TAK1 and IKK activation, NF- κ B activation and ligand-independent osteoclast differentiation [14]. Collectively, our data supports the hypothesis that TRAF6 auto-ubiquitination is the critical upstream mediator of IKK activation.

In this study, we addressed the functional role of the Ub ligase activity of TRAF6 in RANK signaling. We show that TRAF6, but not an E3 Ub ligase defective TRAF6 is able to rescue RANKL-dependent IKK activation and osteoclast differentiation in TRAF6-deficient monocytes. In support of the requirement of TRAF6 Ub ligase activity in RANK signaling, a catalytically inactive Ubc13 or stable knockdown of Ubc13 expression results in suppression of RANK-mediated TRAF6 ubiquitination, IKK and JNK activation, and osteoclast differentiation. Additionally, RANKL stimulated TRAF6 ubiquitination is linked via Lys63 poly-Ub chains and is not targeted for degradation. These findings therefore document an essential role of the TRAF6 RING-dependent Ub ligase activity in RANK signaling in osteoclast progenitors.

Materials and methods

Cell Lines, Reagents, and Antibodies

The mouse macrophage cell line RAW264.7 (referred to as RAW) and HEK293 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and cultured as previously described [8,9,15]. Retroviral packaging cell line GP2-293 was purchased from Clontech (Palo Alto, CA). For primary monocytes, spleens were excised from 6–8 week old C57BL/6 mice and the monocytes were cultured as previously described [14]. The mice were housed in a temperature-controlled environment with free access to food and water. All the protocols and procedures were approved by the Institutional Animal Care and Use Committee at The University of MD Anderson Cancer Center.

Monoclonal antibodies to phospho-I κ B α and polyclonal antibody to phospho-c-Jun were purchased from New England Biolabs (Ipswich, MA); goat anti-rabbit IgG-conjugated horseradish peroxidase from BioRad Laboratories; rabbit polyclonal antibodies against TAB2, TAK1, JNK1, I κ B α , NEMO, and TRAF6 and monoclonal antibodies for TRAF6 and Ub from Santa Cruz Biotechnology (Santa Cruz, CA); goat anti-mouse IgG-conjugated horseradish peroxidase from BD Biosciences Pharmingen; rabbit polyclonal antibody against mouse TRAF6 and mouse monoclonal antibody against alpha-tubulin from CalBiochem; rabbit polyclonal antibody to beta-actin from Cytoskeleton (Denver, CO); and mouse monoclonal antibody for Ubc13 from Zymed (San Francisco, CA). Monoclonal antibody to HA was a generous gift from Dr. G. B. Mills (UTMDACC). Monoclonal antibodies for RANK and IKK β were a generous gift from Dr. S. Singh (Imgenex; San Diego, CA). Monoclonal anti-FLAG, MG132, N-ethyl-maleimide (NEM), and a tartrate resistant acid phosphatase (TRAP) kit were purchased from Sigma. Lys63 and Lys48 poly-Ub chains were purchased from Boston Biochem (Cambridge, MA). Recombinant mouse RANKL, GST-cJun(1–79), GST-I κ B α (1–54), GST-TAB2 and GST-TRAF6 were purified essentially as described [8,15,16].

Plasmids

Expression vectors for FLAG-TRAF6, FLAG-TRAF6-C70A, HA-Ub, HA-JNK, and FLAG-Ubc13-C87A were described previously [8,14,16,17]. The retroviral expression vector pMX-IRES-GFP-puro has been previously described [14]. Human TAB2 encoding residues 574–693 with or without a Zn-finger mutation (C670/673A) was cloned into pGEX-4T1. All site-directed mutagenesis was performed using the QuikChange kit (Stratagene) and verified by DNA sequencing.

Transfection, Reporter Gene Assays and Retroviral Infection

Transfection of HEK293 cells was performed essentially as described [8,14]. Production of retroviral supernatants from GP2-293 cells and infection of spleen-derived monocytes cells was previously described [14].

Osteoclast Differentiation

For osteoclast differentiation of RAW clones, cells were plated in 24-well plates, stimulated the next day with RANKL, and on day 4 or 5, cells were fixed and stained for TRAP. For osteoclast differentiation of primary monocytes, BMMs were isolated from 5-day old femurs of TRAF6-deficient mice and cultured in α -MEM (10% serum) with 5% L929-M-CSF conditioned medium as previously described [14]. When the density of the monocytes was sufficient for the experiment, the cells were washed in phosphate-buffered saline (PBS), lifted with versene buffer, counted, and seeded in 96-well plates. The next day the cells were infected with the indicated retrovirus for 2 days followed by stimulation in the presence of RANKL (100 ng/ml) and 5% L929-M-CSF until osteoclast appeared (4 to 5 days) and then stained for TRAP.

RNA Interference

Four different shRNA sequences against mouse Ubc13 cloned in the pRetroSuper-puro (pRS) vector were purchased from Origene Technologies (Rockville, MD). The sequence corresponding to the catalog number TI594634 was most effective to down-regulate expression of Ubc13 in RAW cells. To establish RAW clones with down regulated expression of Ubc13, RAW cells were infected with retroviral supernatant as described previously [14] and following puromycin selection for 2 days, the cells were trypsinized, counted, and seeded in 96-well plates to isolate single cell clones. Selected clones were evaluated for expression of Ubc13 by western blotting.

GST-fusion Pull-down Assays

Purified GST-TAB2-WT-(574–693) or a Zinc finger mutant GST-TAB2-ZnFm-(574–693) protein were used to pull down linkage-specific poly-ubiquitin chains essentially as described [18]. Briefly, GST, or the indicated GST-fusion proteins bound to glutathione-agarose were incubated with either Lys63 or Lys48 poly-Ub chains (500 ng) or cell lysates in binding buffer (20 mM TRIS, pH 7.4, 150 mM KCl, 0.5% NP40, 1 mM EGTA, 2 mM MgCl₂, and 1 mM DTT) supplemented with 0.1% BSA for 1 h at 4°C with end-over-end rotation. The proteins were washed 3 times in binding buffer followed by one wash in low salt buffer, boiled in SDS-sample buffer for 5 min, subjected to SDS-PAGE, and immunoblotted with anti-Ub.

Western Blotting, Immunoprecipitation, and In Vitro Kinase Assays

Cells were left unstimulated or stimulated as indicated in the figure legends and washed two times with PBS. Lysates were prepared and immunoprecipitation, *in vitro* kinases assays, and immunoblotting was performed as previously described [14,19].

Results and discussion

TRAF6-C70A Cannot Rescue RANKL-dependent Signaling in TRAF6-deficient Monocytes

Our previous study indicated that ectopic expression of TRAF6, but not a RING domain mutant (TRAF6-C70A) was able to activate IKK and JNK in TRAF6-deficient mouse embryonic fibroblasts (MEFs) [14]. Additionally, expression of TRAF6, but not the RING domain mutant supported spontaneous osteoclast formation in TRAF6-deficient monocytes [14], however the number of osteoclasts were fewer than observed with RANKL-treatment of normal monocytes. While these data provide evidence that the TRAF6 RING domain is necessary for signaling, they do not demonstrate that the RING domain is required for RANKL-induced signaling. Thus, to determine whether TRAF6-C70A could rescue RANKL-mediated events in TRAF6-deficient osteoclast progenitors, spleen-derived monocytes from TRAF6-deficient mice were infected with pMX, TRAF6, and TRAF6-C70A. RANKL stimulation of IKK activity and detection of phospho-I κ B α was significantly impaired in cells infected with either pMX or TRAF6-C70A as compared with cells infected with TRAF6 (Fig. 1A). Similar to our previous study in TRAF6-deficient MEFs, over expression of TRAF6, but not TRAF6-C70A was sufficient to induce phospho-I κ B α and IKK activation (Fig. 1A; compare lanes of time zero). In addition, BMMs from TRAF6-deficient mice infected with TRAF6, but not empty vector or TRAF6-C70A was able to rescue TRAP⁺ multi-nucleated osteoclast differentiation after RANKL treatment (Fig. 1B). These data support an essential role of the Ub ligase activity of TRAF6 for RANKL-induced signaling and osteoclast differentiation.

RANK Induces Ubiquitination of Endogenous TRAF6 that is Dependent on Ubc13

Since TRAF6 is the primary signaling adaptor in RANK signaling and utilizes the dimeric E2 enzyme Ubc13/Uev1A for its Ub ligase activity, we next examined whether a catalytically inactive Ubc13 inhibits RANK-induced TRAF6 ubiquitination. While co-transfection of RANK and HA-Ub in HEK293 cells induced ubiquitination of endogenous TRAF6 (Fig. 2A), increased expression of a catalytically inactive mutant of Ubc13 (Ubc13-C87A) prevented this ubiquitination (Fig. 2A, **left panel**). In contrast, expression of wild type Ubc13 did not interfere with RANK-induced TRAF6 ubiquitination (Fig. 2A, **right panel**). Additionally, RANK-induced NF- κ B luciferase reporter activity and JNK activation was significantly impaired when co-transfected with Ubc13-C87A in HEK293 cells (Fig. 2B). These results suggest that RANK-induced ubiquitination of TRAF6 and its ability to activate NF- κ B and JNK depends on catalytically active Ubc13.

RANKL Signaling is Attenuated by Down-Regulation of Ubc13

Having established that TRAF6 auto-ubiquitination is dependent on its RING domain and Ubc13, we next asked whether down-regulation of Ubc13 in an osteoclast progenitor would interfere with RANKL signaling. To explore this hypothesis, we selected a retroviral system to stably knockdown Ubc13 using an shRNA strategy. Following selection with puromycin, single cell RAW clones in which Ubc13 expression was down-regulated as compared with RAW clones infected with the empty vector (pRS) were examined. As compared to two clones infected with the empty vector, we identified two clones with a dramatic reduction of Ubc13 expression and two clones with a mild reduction of Ubc13 expression (Supplementary Fig. S1A). Since each of the cell lines expressed similar amounts of RANK, TRAF6, TAB2, TAK1, NEMO, IKK β , and JNK, the reduction of RANKL signaling in the Ubc13 knockdown clones was not due to the absence of these key molecules involved in RANKL signaling (Supplementary Fig. S1B). Further characterization of two of these clones by a time course experiment with RANKL indicated a reduction of both IKK and JNK activation in cells with reduction of Ubc13 (Fig. 3A). Furthermore, knockdown of Ubc13 also resulted in reduction of phospho-I κ B α , which correlated with mild degradation of I κ B α after RANKL stimulation (Fig. 3A). Significantly, RANKL-mediated TRAF6 ubiquitination was severely impaired in

Ubc13 knockdown cells (Fig. 3B). Additionally, knockdown of Ubc13 prevented RANKL-mediated osteoclast differentiation (data not shown). Collectively, our data provide clear evidence that Ubc13 plays an important role in RANKL signaling leading to TRAF6 auto-ubiquitination and IKK and JNK activation.

RANKL Stimulates Lys63-linked Ubiquitination of TRAF6

To address the nature of TRAF6 ubiquitination induced by RANKL in an osteoclast progenitor, we exploited the specificity of the TAB2 C-terminal Zn-finger domain for Lys63-linked poly-Ub chains [20]. Therefore, we constructed a GST-fusion protein consisting of the C-terminal domain of TAB2 (residues 574–693) and established that this fusion protein, but not its Zn-finger mutant, was able to bind Lys63-linked poly-Ub chains while exhibiting no affinity for Lys48-linked poly-Ub chains (Fig. 4A). We then examined the ability of GST-TAB2 or its Zn-finger mutant to interact with endogenous TRAF6 from RANKL stimulated RAW cells. GST-TAB2, but not its Zn-finger mutant, was capable of binding TRAF6 from RANKL treated RAW cells (Fig. 4B). Furthermore, higher molecular weight TRAF6 species were observed in the GST-TAB2 pull down that appear in a RANKL-dependent manner, which suggest primarily Lys63-linked poly-ubiquitination of TRAF6 by RANKL. The affinity of GST-TAB2 for TRAF6 after RANKL stimulation is time dependent and is maximal with the appearance of poly-ubiquitinated form of TRAF6, which may suggest that recruitment of TAB2 to TRAF6 is poly-Ub dependent. TRAF6 in the presence of Ubc13/Uev1A facilitates Lys-63-linked ubiquitination of itself and other downstream targets, which is different from Lys-48-linked ubiquitination that targets proteins for proteasomal degradation [14]. Consistent with this proposition, we failed to observe any degradation of TRAF6, TAB2, and NEMO after RANKL stimulation of either RAW cells or primary monocytes (Supplementary Fig. S2), which implies that this ubiquitin modification is not Lys48-linked and does not lead to protein degradation.

Conclusions

In RANKL signaling, the cytoplasmic domain of RANK recruits TRAF6 to initiate a signaling cascade that is crucial for the maturation of monocyte precursors to fully differentiated osteoclasts. In this study, we addressed the posttranslational modification associated with TRAF6 following RANKL stimulation in the context of osteoclast differentiation. We demonstrated that ectopic expression of TRAF6, but not TRAF6-C70A was able to rescue IKK activation and osteoclast differentiation after RANKL stimulation in TRAF6-deficient monocytes. To address the biological relevance of Ubc13 in RANK signaling, we first demonstrated that a catalytic mutant of Ubc13 was able to block RANK-induced TRAF6 ubiquitination and NF- κ B and JNK activation. In confirmation of these results, stable knockdown Ubc13 expression in an osteoclast progenitor provided evidence that significant loss of Ubc13 correlated with a decrease in RANKL responsiveness. We next used the specificity of the Zn-finger domain of TAB2 for Lys63 poly-Ub chains to demonstrate that poly-ubiquitination chain associated with TRAF6, following RANKL stimulation is Lys63-linked. Taken together, these data support the critical role of the Ub ligase activity of TRAF6 in RANK signaling and osteoclast differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to generously thank Dr. Singh for providing us with RANK, TRAF6, and NEMO antibodies; Dr. Kitamura for providing the pMX vectors; and Dr. Mills for providing the anti-HA antibody. The DNA Sequencing Core facility

is funded in part through a grant from the NCI (CA16672DAF). This work was supported in part by institutional start-up funds (B. G. D.).

The abbreviations used are

IP, immunoprecipitation
 IB, immunoblot
 BMM, bone marrow-derived monocyte
 JNK, c-Jun N-terminal kinase
 MAPK, mitogen-activated protein kinase
 TNF, tumor necrosis factor
 TAB1, 2, 3, TAK1 binding protein 1, 2, 3
 GST, glutathione *S*-transferase
 DTT, 1,4-Dithiothreitol
 GFP, green fluorescent protein
 TRAF, TNF receptor-associated factor
 TGF- β , transforming growth factor β
 TAK1, TGF- β -activated kinase 1
 IKK, I κ B kinase
 RANK, receptor activator of NF- κ B
 WT, wild type
 HEK, human embryonic kidney
 NF- κ B, nuclear factor- κ B
 ERK, extracellular signal-regulated kinase
 RANKL, RANK ligand
 TRAP, tartrate-resistant acid phosphatase
 RING, really interesting new gene
 SDS, sodium dodecylsulfate
 PBS, phosphate-buffered saline
 Ub, ubiquitin.

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Fig. 1. A TRAF6 RING domain mutant cannot rescue RANKL-induced signaling in TRAF6-deficient monocytes

(A) TRAF6-C70A cannot rescue RANKL-induced signaling in TRAF6-deficient monocytes. Spleen-derived monocytes were isolated from TRAF6-deficient mice and infected respectively with pMX, TRAF6, TRAF6C70A for 2 days. The cells were starved overnight and then stimulated with RANKL (100 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with NEMO antibody and an *in vitro* kinase assay was performed (*top panel*). Cell lysates were also immunoblotted with the indicated antibodies (*bottom panels*).

(B) TRAF6, but not TRAF6-C70A, rescues RANKL-mediated osteoclast differentiation in TRAF6-deficient monocytes. BMM cells from TRAF6-deficient mice were infected with the

indicated retrovirus and then stimulated with RANKL (100 ng/ml) until osteoclasts appeared after which cells were stained for TRAP. Pictures were taken from a representative field at 10x magnification.

Fig. 2. Catalytically inactive Ubc13 prevents RANK-induced TRAF6 ubiquitination and RANK signaling

(A) A catalytic mutant of Ubc13 (Ubc13-C87A) inhibits RANK-mediated ubiquitination of TRAF6. HEK293 cells were co-transfected with empty vector or RANK in the absence (-) or presence (+) of either FLAG-Ubc13-C87A (*left panels*) or Ubc13 (*right panels*) together with HA-Ub. Cell lysates were immunoprecipitated with anti-TRAF6 and the ubiquitination of TRAF6 was determined by immunoblotting with anti-HA. The membrane was stripped and reprobed with anti-TRAF6. Cell lysates were also immunoblotted with the indicated antibodies (*bottom*). (B) Ubc13-C87A blocks RANK signaling. HEK293 cells were co-transfected with empty vector or RANK with increasing amounts of Ubc13-C87A together with an NF-κB-

luciferase reporter and HA-JNK1. Thirty six hours after transfection, cells were harvested and a fraction of the lysate was used to measure luciferase activity (*left*) and another portion of the lysate was used to measure JNK activation by immunoprecipitating with anti-HA followed by an *in vitro kinase* assay with GST-cJun (*bottom right*). Cell lysate was also immunoblotted with the indicated antibodies (*top right*).

Fig. 3. Stable knockdown of Ubc13 expression impairs RANKL signaling in RAW cells

(A) Attenuation of RANKL-mediated JNK and IKK activation by knockdown of Ubc13. The indicated RAW clones were stimulated with RANKL (100 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with JNK1 and NEMO antibodies respectively, and *in vitro* kinase assays were performed (*upper panels*). Cell lysate were also immunoblotted with the indicated antibodies (*bottom panels*). (B) Reduction of RANKL-mediated TRAF6 ubiquitination by knockdown of Ubc13. The indicated RAW clones were treated with RANKL (100 ng/ml) for the various times and lysed in Buffer A and immunoprecipitation of endogenous TRAF6 was performed in Buffer C as previously described [14]. Bound proteins were subjected to SDS-PAGE and immunoblotted with anti-Ub (*top panel*), and then the membrane was stripped and reprobed with anti-TRAF6 (*middle panel*). Cell lysates were also immunoblotted with the indicated antibodies (*bottom panels*). HC, Heavy Chain.

Fig. 4. TRAF6 is ubiquitinated by Lys63 Ub chains and is not down regulated after RANKL stimulation

(A) TAB2 selectively binds Lys63-linked Ub chains. GST or the indicated GST-fusion proteins linked to glutathione-agarose beads were mixed with Lys63 or Lys48 poly-Ub chains and pull-down assays were performed. Bound proteins were eluted in SDS-sample buffer and subjected to SDS-PAGE, and immunoblotted with anti-Ub (*left panel*) and 10% of the input is shown (*right panel*). (B) TAB2-WT but not TAB2-ZnFm mutant, interacts with endogenous TRAF6. RAW cells stimulated with RANKL (100 ng/ml) for the indicated times were lysed in Buffer A and mixed with the indicated GST-fusion proteins in Buffer B and processed as described earlier [14]. The membrane was stripped and reprobed with anti-TRAF6 (two exposures are shown); then the membrane was stained with Ponceau S (*bottom*).