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Identification of a consensus site for TRAF6/p62 polyubiquitination

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Abstract

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is an ubiquitin ligase that regulates a diverse array of physiological processes via forming Lys-63 linked polyubiquitin chains. In this study, the lysine selection process for TRAF6/p62 ubiquitination was examined. The protein sequence of two characterized TRAF6/p62 substrates, NRIF and TrkA, revealed a conserved consensus pattern for the ubiquitination site of these two TRAF6 substrates. The consensus pattern established in the verified substrates was common to the other Trk receptor family members, TrkB and TrkC. Interestingly, Lysine 811 in TrkB was selected for ubiquitination, and mutation of Lysine 811 diminished the formation of TRAF6/p62 complex that is necessary for effective ubiquitination. Moreover, downstream signaling was affected upon binding of BDNF to the mutant TrkB receptor. These findings reveal a possible selection process for targeting a specific lysine residue by a single E3 ligase and underscore the role of the scaffold, p62, in this process.

Keywords

Ubiquitination; Ub ligase; TRAF6; p62; Trk

Many adaptors have been identified in studies of other neuronal tyrosine kinases that may also prove to function in Trk receptor-mediated signaling [1]. Cytoplasmic protein p62 was identified as an interacting partner of atypical protein kinase C (PKC) [2] and has been shown to contain several protein-protein interacting modules that enable the protein to serve as a scaffold for activation of the transcription factor NF- κ B [3]. The multidomain protein structure of p62 is suggestive of diverse protein-protein interactions and its link in cellular functions. The functional motifs in p62 include a Phox and Bem1p domain (PB1) domain that embeds an octicosapeptide Phox, Cdc and the atypical PKC-interaction domain (AID) (OPCA) motif, a ZZ zinc finger, a binding site for Tumor necrosis factor Receptor-Associated Factor 6 (TRAF6), two PEST sequences, and an Ubiquitin-associated (UBA) domain [4]. The C-terminal ubiquitin-associated domain (UBA) was discovered to bind non-covalently to ubiquitin [5]. In vitro binding studies have unveiled p62 as a unique ubiquitin-binding protein, which binds polyubiquitin non-covalently through its C-terminus [6].

Ubiquitination of eukaryotic proteins regulates a broad range of cellular processes. E3 Ub ligases are known to interact with specific substrates either directly or through adaptor proteins.

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In this regard, p62 has been shown to act as an adaptor and interacts with the TRAF domain of TRAF6, resulting in its auto-activation [7,8]. Recent findings from our lab have revealed that both TrkA [9] and the neurotrophin receptor interacting factor (NRIF) [10] are K63-polyubiquitinated by the TRAF6/p62 complex. Mutation analyses of these proteins identified a single acceptor lysine residue that serves as the recognition site for polyubiquitination. TRAF6 possesses a RING finger domain that is responsible for its E3 ligase activity [11]. The E3 ligase binds its substrates through its RING domain, which then mediates polyubiquitination of target proteins. TRAF6, together with E2 UBc1/Uve1A, functions as an E3 ligase to mediate the synthesis of K63 linked polyUb chains [12].

There are only a few other reports on TRAF6-mediated polyubiquitination that include TRAF6 auto-ubiquitination [13], NEMO [13], TAB2 and TAB3 [14]. High substrate specificity of the E3-ubiquitin ligase ensures correct transmission of signals. Yet, little is known about how the substrates are recognized by E3 Ub ligases; nor how site-specific ubiquitination is achieved, and more specifically, why one lysine may be preferred over the other. In the current study, we investigated this selection process. We examined the protein sequence of the verified TRAF6/p62 substrates and identified a consensus pattern therein. This sequence was then used to screen the protein sequence of the other members of the family of Trk receptor proteins. Employing our bioinformatics predictions a primary ubiquitination site in TrkB and predicted site in TrkC was identified.

Materials and methods

Antibodies

The mouse ubiquitin, HA and p62, rabbit Trk (C-14), HA, and TRAF6 antibodies were purchased from Santa Cruz Biotechnology, La Jolla, CA. Phospho- and nonphospho-MAPK antibodies were purchased from New England Biolabs, and rabbit antibody to phospho Akt (Ser 473), and non-phospho Akt were obtained from Cell Signaling (Beverly, MA). 2.5 S nerve growth factor (NGF), BDNF and NT3 were purchased from Bioproducts for Science (Indianapolis, IN).

Cell Culture

Human embryonic kidney (HEK) 293 and nnr5 cells were grown as previously described [8]. HEK293 cells were transfected with the calcium phosphate method by using a Mammalian Cell Transfection Kit (Specialty Media), and nnr5 cells were transfected by using LipofectAMINE 2000 (Invitrogen Life Technologies). The cells were lysed with Triton lysis buffer to detect protein-protein interactions (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM NaF, 0.5% Triton X-100, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 2 µg/ml leupeptin and aprotinin) or SDS lysis buffer to detect covalent interaction of ubiquitin and TrkA (Triton lysis buffer containing 1% SDS) [6]. Protein was estimated by Bradford procedure (Bio-Rad) and with bovine serum albumin (BSA) as a standard for all samples except those containing SDS, which were estimated by DC assay (Bio-Rad).

Immunoprecipitation and Western Blotting Analysis

Cell lysates (1 mg) were diluted in lysis buffer and incubated with 4 µg of primary antibody at 4°C for 3 hr. The immunoprecipitates were collected with agarose-coupled secondary antibody for 2 hr at 4°C and then were washed three times with lysis buffer. The samples were boiled in sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on gels, transferred onto nitrocellulose membranes, and analyzed by Western blotting with the appropriate antibodies. The samples were separated by 7.5% SDS-PAGE and probed with ubiquitin or TrkA antibodies.

Site-Directed Mutagenesis

All primers were obtained from Integrated DNA Technologies, Inc. Coralville, IA and were used without further purification. The forward 32-base primer used to generate the mutant was 5' CTTCAGAACTTGGCGAGGGCGTCGCCCGTCTAC 3' and the reverse primer was 3' GTAGACGGGCGACGCCCTCGCCAAGTTCTGAAG 5'. QuickChange II XL Site-Directed Mutagenesis Kit was used according to the manufacturer's standard protocol (Stratagene, La Jolla, CA) to mutate A → G at position 3096 resulting in K → R amino acid change in rat TrkB protein sequence (NM_012731.1). The presence of the correct mutation and the absence of PCR-derived alterations to the coding sequence were confirmed by completely sequencing of the mutant receptor construct.

Results and discussion

Conserved sequences flanking the TRAF6/p62 ubiquitin acceptor site

Two independent reports have identified TrkA [9] and NRIF [10] as TRAF6/p62 substrates. Moreover, the specific lysine residue in both these proteins that serve as the ubiquitin acceptor site was identified. Therefore, in an effort to examine similarities in the lysine selection process for substrate ubiquitination by TRAF6/p62 the protein sequence of TrkA and NRIF was examined. A similarity in the sequences between these two proteins around their primary ubiquitination site revealed a conserved pattern based on chemical properties of the amino acids of the flanking residues at the acceptor lysine. The consensus pattern observed was [—(hydrophobic)—K—(hydrophobic)—X—X—(hydrophobic)—(polar)—(hydrophobic)—(polar)—(hydrophobic)] where K is the ubiquitinated lysine residue and X any other amino acid (Fig. 1).

Both TrkB and TrkC were examined to determine if this consensus pattern existed in the other members of the Trk family, since both TrkB and TrkC have been reported to be ubiquitinated by TRAF6/p62 [9]. Interestingly, Lysines at 811 in TrkB and 602 and 815 in TrkC possessed a similar pattern in their flanking amino acids homologous to the sequence observed in TrkA and NRIF (Fig. 1). Therefore, we hypothesized that these lysines might act as primary ubiquitin acceptor sites. To test our hypothesis, we focused on the TrkB receptor, since it possessed only one putative ubiquitin acceptor site at K811. In order to test the possibility that this Lysine was a putative ubiquitin acceptor site, we replaced Lysine at 811 with Arginine (K811R) using site-directed mutagenesis and generated a mutant receptor. The mutant was verified by sequencing. In addition, absence of other mutations was verified by sequencing the entire TrkB cDNA. Secondary structure analysis of protein sequence at an online protein structure prediction server PSIPRED (<http://bioinf.cs.ucl.ac.uk/psipred/>) revealed that the ubiquitinated Lysines, K485 of TrkA and K19 of NRIF assumed a coiled-coil motif and K811 in TrkB was likewise predicted to be in a coiled-coil region.

TRAF6 ubiquitinates TrkB at Lysine 811

To check for efficient detection of ubiquitinated wild-type TrkB, HEK293 cells were co-transfected with the HA-tagged TrkB (Wild-type (WT) and Mutant) and the His/myc-tagged Ubiquitin. As control, ubiquitination of WT-TrkA and K485R TrkA mutant [9] was examined. Maximum polyubiquitination of Trk receptors has been observed after 15 min treatment with neurotrophins [15]. Post-transfection HEK cells were treated with their respective neurotrophin, NGF or BDNF, for 15 min and the extent of receptor ubiquitination was determined by immunoprecipitation with Trk antibody and Western blotting with anti-ubiquitin (Fig. 2, upper panel). TrkA was polyubiquitinated upon addition of NGF and ubiquitination was significantly diminished by mutating K485R (Fig. 2, compare lanes 3 and 5). Likewise, TrkB was polyubiquitinated upon addition of BDNF, while mutation at K811R significantly impaired receptor ubiquitination (Fig. 2, compare lanes 7 and 9). In addition, a

fraction of lysate was blotted with Trk antibody to verify the expression levels of all the constructs (Fig. 2, lower panel). Consistently diminished expression of TrkB was observed when K811 was mutated to R, suggesting that this lysine may regulate turnover of the protein. K811 is a primary ubiquitination site, however, a residual amount of polyubiquitin signal was observed on the blot, which might be due to the presence of an additional lysine residue(s) that is also ubiquitinated. Altogether, these results demonstrate that K811 is a preferential/primary ubiquitin acceptor site in TrkB.

Mutation impairs p62's ability to link TrkB to TRAF6

P62 serves as an adaptor bridge to recruit TRAF6 through its TRAF6 binding site [3,8,9]. Therefore, studies were undertaken to examine whether mutation at the primary ubiquitination site in the TrkB receptor impairs formation of a TRAF6/p62 signaling complex. HEK cells were transfected with WT-TrkA, WT-TrkB or their point mutants K485R-TrkA and K811R-TrkB followed by treatment with neurotrophins, either NGF or BDNF, for 15 min to attain maximum polyubiquitination. The cell extracts were immunoprecipitated with Trk antibody and immunoblotted with Trk antibody as control, and TRAF6 and p62 antibody to examine their presence in the complex (Fig. 3). TRAF6 was detected only in lysates recovered from stimulated cells expressing WT receptors (Fig. 3), along with the p62 adaptor (Fig. 3). TRAF6 and p62 were absent in lysates recovered from cells expressing mutant Trk receptors. These results reveal that mutating the primary ubiquitin acceptor site in either TrkA or TrkB disrupts the interaction between p62, TRAF6 and the Trk receptors (Fig. 3).

Receptor ubiquitination implicated in regulation of TrkB downstream signaling

Ligand binding induces Trk receptors to initiate autophosphorylation. These phosphorylated residues later serve as sites for additional effector factors, and enzymes to bind and propagate the signal downstream. This leads to rapid and sustained activation of various signaling pathways, including the Ras/MAPK pathway and the AKT pathway [16]. The ability of NGF and BDNF to stimulate downstream MAPK and AKT signaling as compared to their mutant counterparts was examined. HA-tagged WT-TrkA, WT-TrkB, or their mutants were transfected in nnr5 cells and treated with either NGF or BDNF for 15 min. The lysates recovered from neurotrophin-treated cells were blotted with phospho-MAPK and phospho-AKT antibody, stripped and reprobed with non-phospho antibodies to each protein (Fig. 4). NGF-induced MAPK and AKT activation in the cells expressing WT receptors was impaired in cells expressing mutant TrkA. BDNF had no effect on MAPK activation in the cells expressing the mutant TrkB receptor. However, mutation of K811R in TrkB induced hyper-activation of AKT. This suggests that despite high degree of sequence similarity and broadly overlapping signaling pathways, there still exists divergent signaling response.

Herein we reveal a conserved motif that serves as a recognition determinant for TRAF6/p62 enzyme complex. Ubiquitination is the second most common post-translational modification and is highly conserved in eukaryotes. The choice of lysine is an important decision as it determines the fate of the protein [17]. Analysis of the available data on ubiquitination sites in yeast showed clear preference for ubiquitination based on structure-function relationship [18]. Some structural preferences exist for ubiquitin ligation of the targeted proteins such as preferred choice of lysines in α -helices, and then for easily accessible lysines in the loop regions. Our findings add to the growing list that indicates a bias towards a consensus sequence motif for ubiquitination by a given E3 [19–25]. Moreover, it appears that the E3 targets an accessible surface residue providing the selection process with a conformational recognition mechanism. The TRAF6-p62 signaling complex leads to autoactivation of TRAF6 [8]. The scaffold, p62, then recruits the substrate enabling the E3 to scan for the easily accessible lysine residues in the loops and helical structures on the surface of the substrate resulting in polyubiquitination at a specific lysine, if the flanking residues fit the consensus motif. This

report provides a strategy for studying how TRAF6 defines its lysine specificity and reveals how scaffolds proteins, on which these complex chemical reactions take place, aid in selecting substrates. Further studies will be needed to develop algorithms and an appropriate search strategy to identify this consensus motif in other TRAF6 and/or p62 interacting proteins.

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References

1. Grimm J, Sachs M, Britsch S, Di Cesare S, Schwarz-Romond T. Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. *J. Cell Biol* 2001;154:345–354. [PubMed: 11470823]
2. Sanchez P, De Carcer G, Sandoval IVJ, Moscat J, Diaz-Meco MT. Localization of Atypical Protein Kinase C Isoforms into Lysosome-Targeted Endosomes through Interaction with p62. *Mol Cell Biol* 1998;18:3069–3080. [PubMed: 9566925]
3. Moscat JM, Diaz-Meco MT, Wooten MW. Signal integration and diversification through the p62 scaffold protein. *Trends Biochem Sci* 2007;32:95–100. [PubMed: 17174552]
4. Geetha T, Wooten MW. Structure and functional properties of the ubiquitin binding protein p62. *FEBS Lett* 2002;512:19–24. [PubMed: 11852044]
5. Mueller TD, Feigon J. Solution structures of UBA domains reveal a conserved hydrophobic surface for protein-protein interactions. *J. Mol. Biol* 2002;319:1243–1255. [PubMed: 12079361]
6. Seibenhener ML, Babu JR, Geetha T, Wong HC, Krishna NR, Wooten MW. Sequestosome 1/P62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. *Mol. Cell. Biol* 2004;24:8055–8068. [PubMed: 15340068]
7. Wooten MW, Seibenhener ML, Mamidipudi V, Diaz-Meco MT, Barker PA, Moscat J. The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. *J Biol Chem* 2001;276:7709–7712. [PubMed: 11244088]
8. Wooten MW, Geetha T, Seibenhener ML, Babu JR, Diaz-Meco MT, Moscat J. The p62 scaffold regulates nerve growth factor-induced NF-kappaB activation by influencing TRAF6 polyubiquitination. *J Biol Chem* 2005;280:35625–35629. [PubMed: 16079148]
9. Geetha T, Jiang J, Wooten MW. Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Mol Cell* 2005;20:301–312. [PubMed: 16246731]
10. Geetha T, Kenchappa RS, Wooten MW, Carter BD. TRAF6-mediated ubiquitination regulates nuclear translocation of NRIF, the p75 receptor interactor. *EMBO J* 2005;22:3859–3868. [PubMed: 16252010]
11. Rothe M, Wong SC, Henzel WJ, Goeddel DV. A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 1994;78:681–692. [PubMed: 8069916]
12. Deng L, Wang C, Spencer E, Yang L, Braun A, You J, Slaughter C, Pickart C, Chen ZJ. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 2000;103:351–361. [PubMed: 11057907]
13. Lamothe B, Besse A, Campos AD, Webster WK, Wu H, Darnay BG. Site-specific Lys-63-linked Tumor Necrosis Factor Receptor-associated Factor 6 Auto-ubiquitination Is a Critical Determinant of IkB Kinase Activation. *J. Biol. Chem* 2007;282:4102–4112. [PubMed: 17135271]
14. Ishitani T, Takaesu G, Ninomiya-Tsuji J, Shibuya H, Gaynor RB, Matsumoto K. Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. *EMBO J* 2003;22:6277–6288. [PubMed: 14633987]
15. Geetha T, Wooten MW. Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling. *J Biol Chem* 2003;278:4730–4739. [PubMed: 12471037]
16. Sudo T, Maruyama MH, Osada H. P62 functions as a p38 MAP kinase regulator. *Biochem Biophys Res Commun* 2000;269:521–525. [PubMed: 10708586]
17. Weissman AM. Themes and variations on ubiquitylation. *Nat Rev Mol Cell Biol* 2001;169–178. [PubMed: 11265246]

18. Catic A, Collins C, Church GM, Ploegh HL. Preferred in vivo ubiquitination sites. *Bioinformatics* 2004;20:3302–3307. [PubMed: 15256413]
19. Petroski MD, Deshaies RJ. Context of multiubiquitin chain attachment influences the rate of Sic1 degradation. *Mol. Cell* 2003;11:1435–1444. [PubMed: 12820958]
20. Ju D, Xie Y. Identification of the preferential ubiquitination site and ubiquitin-dependent degradation signal of Rpn4. *J Biol Chem* 2006;281:10657–10662. [PubMed: 16492666]
21. Galluzzi L, Paiardini M, Lecomte MC, Magnani M. Identification of the main ubiquitination site in human erythroid alpha-spectrin. *FEBS Lett* 2001;489:254–258. [PubMed: 11165260]
22. Wu G, Xu G, Schulman BA, Jeffrey PD, Harper JW, Pavletich NP. Structure of a β -TrCP1-Skp1- β -Catenin Complex Destruction Motif Binding and Lysine Specificity of the SCF $^{\beta$ -TrCP1 Ubiquitin Ligase. *Mol. Cell* 2003;11:1445–1456. [PubMed: 12820959]
23. Scherer DC, Brockman JA, Chen Z, Maniatis T, Ballard DW. Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc. Natl. Acad. Sci. USA* 1995;92:11259–11263. [PubMed: 7479976]
24. Kumar KG, Tang W, Ravindranath AK, Clark WA, Croze E, Fuchs SY. SCF(HOS) ubiquitin ligase mediates the ligand-induced downregulation of the interferon-alpha receptor. *EMBO J* 2003;22:5480–5490. [PubMed: 14532120]
25. Kumar KG, Krolewski JJ, Fuchs SY. Phosphorylation and specific ubiquitin acceptor sites are required for ubiquitination and degradation of the IFNAR1 subunit of type I interferon receptor. *J. Biol. Chem* 2004;279:46614–46620. [PubMed: 15337770]

TrkA_rat -- gkgsglqghi	G	K	G	S	G	L	Q	G	H	I	K485
NRIF_mouse -- vkfedvslf	V	K	F	E	D	V	S	L	T	F	K19
Consensus pattern	*	K	*	X	X	*	!	*	!	*	
<i>Putative sites</i>											
TrkB_rat -- akaspyldi	A	K	A	S	P	V	Y	L	D	I	K811
TrkC_rat --vkfygvcgdp	V	K	F	Y	G	V	C	G	D	P	K602
TrkC_rat -- gkatpiyldi	G	K	A	T	P	I	V	L	D	I	K815

Fig. 1.

Conserved sequences flanking the TRAF6/p62 ubiquitin acceptor site. An alignment of TRAF6/p62 ubiquitination acceptor site in NRIF and TrkA shown here with maximum number of matches. Amino acids of the same typed are marked as (*) hydrophobic; (!) polar, (X) any amino acid residue and (K) the acceptor Lysine residue.

Fig. 2. TrkB is ubiquitinated at Lysine 811. HEK cells were transfected with either WT-TrkA, K485R-TrkA, WT-TrkB or K118R-TrkB along with His/Myc-tagged ubiquitin constructs. The cells were treated with or without, NGF for TrkA; BDNF for TrkB for 15 min. The cells were then lysed with SDS lysis buffer and the extent of ubiquitination was determined by immunoprecipitating the lysate with Trk antibody and Western blotting with anti-ubiquitin (upper panel). As a control, a fraction of lysate (50 μ g) was blotted with anti-Trk (lower panel). This experiment was replicated three independent times with similar results.

Fig. 3.

Mutation of Trk receptors impairs interaction with TRAF6/p62. HEK cells were transfected with WT-TrkA, WT-TrkB or their point mutants K485R-TrkA and K811R-TrkB. The cells were stimulated with or without NGF and BDNF for 15 min. The cells were then lysed in Triton lysis buffer and the cell lysate was immunoprecipitated with Trk antibody and Western blotted with Trk, TRAF6 or p62 antibody. As a control, a fraction of lysate (50 µg) was blotted with anti-Trk and anti-TRAF6 antibody. This experiment was replicated three independent times with similar results.

Fig. 4.

Receptor ubiquitination regulates downstream signaling. HA-tagged WT-TrkA, WT-TrkB, or their mutants were transfected in NNR5 cells and treated with either NGF or BDNF for 15 min. The lysates from transfected cells were blotted with phospho-MAPK and stripped, and reblotted with nonphospho-MAPK antibody as shown. Alternatively, the lysates were also blotted with phospho-AKT and stripped, and reblotted with nonphospho-AKT antibody. The expression of Trk receptors in the lysate was also examined. This experiment was replicated three independent times with similar results.