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Smurf2 is a TRAF2 binding protein that triggers TNF-R2 ubiquitination and TNF-R2-induced JNK activation

Isabelle Carpentier [a,b,1], Beatrice Coornaert [a,b,1], Rudi Beyaert [a,b,*]

[a] Department of Molecular Biology, Ghent University, Technologiepark 927, B-9052 Gent, Belgium
[b] Department for Molecular Biomedical Research, Unit of Molecular Signal Transduction in Inflammation, VIB, Technologiepark 927, B-9052 Gent, Belgium

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ABSTRACT

TRAF2 plays a central role in TNF-induced signalling to NF-κB and JNK/p38 MAPK. To better understand the molecular mechanisms that mediate this dual function of TRAF2, we performed a yeast two-hybrid screening for TRAF2 interacting proteins using the Sos recruitment system. This resulted in the identification of the E3 ubiquitin ligase Smurf2 as a TRAF2 binding protein. TRAF2 overexpression was shown to trigger Smurf2 ubiquitination and the formation of a TNF-R2/Smurf2 complex. Smurf2 on its turn promoted TNF-R2 ubiquitination and the relocalization of TNF-R2 as well as TRAF2 to a detergent-insoluble cell fraction. This was associated with enhanced TNF-R2-induced JNK activation, whereas TNF-R2-induced NF-κB activation remained unaffected. These results suggest an important role for Smurf2 binding to TRAF2 in determining specific signalling outputs of TNF-R2.

Tumor necrosis factor (TNF) plays a key role in inflammation and immunity and exerts its function by binding to two distinct receptors known as TNF-R1 and TNF-R2 [1]. Both receptors can initiate pro-inflammatory gene expression by activating IκB kinases (IKKs) and different mitogen-activated protein kinases (MAPKs), leading to the activation of nuclear factor κB (NF-κB) and several other transcription factors. TNF receptor associated factor (TRAF) 2 binds directly to TNF-R2, whereas TRADD mediates the binding of TRAF2 to TNF-R1. Mouse gene targeting studies have shown that TRAF2 is indispensable for TNF-induced activation of c-Jun N-terminal Kinase (JNK) and NF-κB [2]. TRAF2 functions as a docking molecule for the recruitment of several kinases such as RIP1 and MAPK kinase kinases that are implicated in the activation of IKK and JNK, respectively [2]. In addition, TRAF2 is a RING E3 ubiquitin ligase for RIP1, whose K63-linked polyubiquitination enables the recruitment of the IKK adaptor protein NEMO [3,4]. In addition, auto-ubiquitination of TRAF2 has been shown to induce its relocalization to an insoluble membrane fraction where it specifically activates JNK [5].

Smurf1 and Smurf2 are HECT E3 ubiquitin ligases that control the transforming growth factor (TGF) β signalling pathway and have been implicated in the degradation of different Smad signalling proteins [6]. Smads function as adapters that recruit Smurfs to the TGF-β receptor complex and the transcriptional repressors SnoN and Ski, thereby also regulating the degradation of these Smad associating proteins [7]. In addition, Smurfs were shown to target several other proteins such as RhoA [8] and β-catenin [9].

In this study, we show that Smurf2 can form a ternary complex with TNF-R2 and TRAF2. Moreover, we show that Smurf2 can trigger TNF-R2 ubiquitination and the redistribution of TNF-R2 to an insoluble cell fraction, thus promoting the specific activation of JNK.

Materials and methods

Cell lines and reagents. HEK293T cells were grown in DMEM with 10% fetal bovine serum. The TNF-R2 specific monoclonal utr-1 antibodies were obtained from Dr. W. Lesslauer (Roche Basel, Basel, Switzerland), polyclonal anti-TNF-R2 from Dr. W. Buurman (University of Maastricht, the Netherlands), monoclonal anti-Myc from Dr. N. Mertens (DMBR, Ghent University—VIB, Ghent, Belgium). Anti-HA was from CRP (Richmond, CA, USA), anti-TRAF2 (sc-876) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), phospho-SAPK/JNK (Thr183/Tyr185) antibody from Cell Signalling Technology (Beverly, MA, USA), anti-GFP (green fluorescent protein) (JL-8) from Clontech (Palo Alto, CA, USA), anti-Flag and anti-Flag-horseradish peroxidise (HRP) from Sigma (St. Louis, MO, USA), anti-E tag and anti-E-HRP from GE Healthcare, Buckinhamshire, UK).

Expression plasmids. Sources of plasmids were: pRK-HA-Smurf2 and pRK-Myc-Smurf2-C/G (Y. Zhang, NCI, Bethesda, MD, USA),

* Corresponding author. Address: Department of Molecular Biology, Ghent University, Technologiepark 927, B-9052 Gent, Belgium. Fax: +32 9 3313609.
E-mail address: rudi.beyaert@dmbr.ugent.be (R. Beyaert).
[1] These authors contributed equally to this work.

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pCMV5B-Flag-Smurf2 WT and deletion mutants (J. Wrana, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada), pcDNA1-TNF-R2 WT and pcDNA1-TNF-R2-ΔT2BD (W. Declercq, Ghent University-VIB, Ghent, Belgium), pEF6-E-TRAF2 WT or ΔRING (B. Depuydt, Ghent University-VIB), pJNK1-Flag (C. Widmann, Denver, CO, USA), pNFconluc (A. Israel, Institut Pasteur, Paris, France), pUT651 (Eurogentec, Seraing, Belgium), pcDNA3-HA-ubiquitin (K. DiMarco-Burns, University of Lausanne, Switzerland), pRK5-mTRAF2 (D. Goeddel, Tularik Inc., CA, USA). pRK-Myc-Smurf2 WT was obtained as follows: pRK-HA-Smurf2 WT and pRK-Myc-Smurf2-C/G were cut with ScaI and the resulting Myc-containing fragment was ligated to the Smurf2 WT containing fragment resulting in pRK-Myc-Smurf2 WT. pSos-mTRAF2 was obtained by PCR on pRK5-mTRAF2 with forward (5′-cgtagcggatccagatggctgcagccagtgtg-3') and reverse (5'-gctgttggcgcgctagagtcctgtt aggtcc-3') primers. Corresponding PCR products were cloned into BamHI/BssHII sites of pSos. The oligo-dT human spleen Cytotrap two-hybrid cDNA-library (# 975207), XhoI/EcoRI inserted into pMyr, and the yeast strain cdc25-2 (genotype MATα *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3 112 cdc25-2 Gal*$^{+}$) (# 217438) were obtained from Stratagene (La Jolla, CA, USA).

Yeast two-hybrid screening. The Sos recruitment two-hybrid system was used. pSos-mTRAF2 was introduced into the cdc25-2 yeast strain. cDNA library transformation (pMyr vector) and the selection of TRAF2 interaction partners was performed as previously described [10].

Transfection, co-immunoprecipitation, and Western blotting. HEK293T cells (1.2×10^6) were plated on 10-cm Petri dishes and transiently transfected with a total amount of 5 μg DNA using the DNA calcium phosphate coprecipitation method. Thirty-six hours later, cells were lysed in the indicated lysis buffer supplemented with protease inhibitors (10 μg/ml leupeptin, 200 U/ml aprotinin and 1 mM PMSF) and phosphatase inhibitors (10 mM NaF, 1 mM Na-vanadate and 5.5 mg/ml β-glycerophosphate). The lysis buffers used were: Smurf2 buffer (10 mM Hepes pH 7.9, 300 mM NaCl, 0.1 mM EGTA, 20% glycerol, 0.2% NP-40), TNF-R2 buffer (10 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 1% NP-40) and RIPA buffer (1% NP-40, 0.1% SDS, 0.5% deoxycholate, PBS). Cell lysates were incubated with the indicated antibodies and immobilized to protein A trisacryl beads (Pierce Chemicals, Rockford, IL, USA). Beads were washed 6 times with buffers as indicated in figure legends, binding proteins were eluated with Laemmli buffer and analyzed by immunoblotting.

Ubiquitination assay. HEK293T cells (1.2×10^6) were transiently transfected with HA-ubiquitin and indicated expression plasmids. The next day, cell extracts were prepared and specific proteins were immunoprecipitated. Beads were washed 6 times in RIPA buffer in case of Flag immunoprecipitation or twice with TNF-R2 lysisbuffer, twice with TNF-R2 lysisbuffer containing 0.75 M NaCl and twice with TNF-R2 lysisbuffer in case of TNF-R2 or E-TRAF2 immunoprecipitation. Bound proteins were eluted with Laemmli buffer and analyzed by immunoblotting with anti-HA to detect ubiquitin conjugates.

Cell fractionation assay. HEK293T cells (2×10^5) were transiently transfected with 100 ng TNF-R2 with or without 1 μg HA-Smurf2 or Myc-Smurf2-C/G. Forty-eight hours later, cells were lysed in 100 μl RIPA buffer for 15 min at 4 °C, followed by centrifugation for 10 min at 4 °C at 14000 rpm in an Eppendorf centrifuge. One-hundred microliters of 2× Laemmli was added to the supernatant fractions, whereas cell pellets were washed with lysis buffer and resolved in 200 μl Laemmli buffer. Total cell lysates were obtained by immediate lysis of cells in 200 μl Laemmli buffer. Fifty microliters of each fraction was used for SDS–PAGE followed by immunoblotting.

NF-κB reporter gene assay and JNK activation assay. NF-κB dependent luciferase reporter assays were performed as previously described [11]. For the JNK activation assay, 2×10^5 HEK293T cells in 6-well plates were transiently transfected by DNA calcium phosphate coprecipitation. Twenty-four hours later, cells were lysed in Laemmli buffer and separated by 10% SDS–PAGE, followed by immunoblotting.

Results

Smurf2 is a novel TRAF2 binding protein

TRAF2 is a central mediator in TNF signalling and is located at a branch point from which NF-κB and MAPK pathways bifurcate. To further understand the mechanisms that regulate this dual function of TRAF2, we decided to screen a human spleen cDNA library for TRAF2 binding proteins using yeast two-hybrid. This resulted in the identification of known (e.g. TRAF1, TRADD, CD40), as well as novel TRAF2 interacting proteins. One newly identified protein corresponded to a fragment of the E3 ubiquitin ligase Smurf2. This clone (amino acids 385–748) contained nearly the whole HECT domain (amino acids 368–748) (Fig. 1A). TRAF2/Smurf2 binding was confirmed by co-immunoprecipitation of transiently transfected HEK293T cells (Fig. 1B). Deletion of Smurf2 WW2 or WW3 domains significantly reduced, whereas deletion of WW1 enhanced, TRAF2 co-immunoprecipitation (Fig. 1C). Most importantly, deletion of the C2 or HECT domain completely abolished the interaction with TRAF2, indicating an absolute requirement of these domains. A E3 ubiquitin ligase inactive Smurf2 mutant (Smurf2-C/G; [12]) bound TRAF2 as efficiently as WT Smurf2 (data not shown). Altogether, these data demonstrate that Smurf2 is a novel TRAF2 binding protein.

TRAF2 triggers the ubiquitination and recruitment of Smurf2 to TNF-R2

The ability of TRAF2 to bind TNF-R2 as well as Smurf2 raised the interesting possibility that TRAF2 functions as an adaptor between Smurf2 and TNF-R2. A similar adaptor function has previously been reported for Smad7, which bridges Smurf1 and Smurf2 to TGFβ-R-I [13,14]. Therefore, we analyzed the effect of TRAF2 overexpression on Smurf2/TNF-R2 co-immunoprecipitation in HEK293T cells. To avoid possible effects of the ubiquitin ligase activity of Smurf2, we used the catalytically inactive Smurf2-C/G mutant in these experiments. TRAF2 co-expression enabled Smurf2/TNF-R2 co-immunoprecipitation, suggesting an adaptor function for TRAF2 (Fig. 2A). However, TRAF2 also enabled co-immunoprecipitation of Smurf2 with TNF-R2-ΔT2BD, in which the TRAF2 binding site was deleted, indicating that TRAF2 enables the formation of a TNF-R2/Smurf2 complex independently of its ability to directly bind TNF-R2.

To further address the mechanism by which TRAF2 might trigger the formation of a Smurf2/TNF-R2 complex, we analyzed its potential to induce Smurf2 ubiquitination. Therefore, we tested the effect of TRAF2 overexpression on the ubiquitination of Smurf2 in HEK293T cells that were transfected with HA-ubiquitin. To avoid a potential contribution of Smurf2 auto-ubiquitination, we used Smurf2-C/A in these experiments. Smurf2 immunoprecipitation followed by immunoblotting with anti-HA revealed that TRAF2 indeed triggered Smurf2 ubiquitination (Fig. 2B). Deletion of the RING finger domain of TRAF2, which disrupts its ubiquitin ligase activity, abolished its ability to induce Smurf2 ubiquitination (Fig. 2B), as well as Smurf2/TNF-R2 complex formation (Supplementary Fig. 1). Altogether, these observations suggest a potential role for TRAF2-mediated Smurf2 ubiquitination in the targeting of Smurf2 to TNF-R2.