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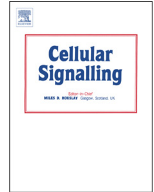

TRAF-mediated modulation of NF-kB AND JNK Activation by TNFR2

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Tumor Necrosis Factor Receptor 2 (TNFR2) activates transcription factor κB (NF-κB) and c-Jun N-terminal kinase (JNK). Most of the biological activities triggered by TNFR2 depend on the recruitment of TNF Receptor-Associated Factor 2 (TRAF2) to the intracellular region of the receptor. The intracellular region of TNFR2 contains five highly conserved amino acid sequences, three of which appear implicated in receptor signaling. In this work we have studied the interaction of TNFR2 with TRAF proteins as well as the functional consequences of this interaction. We show that TRAF1, TRAF2 and TRAF3 bind to the receptor through two different binding sites located at conserved modules IV and V of its intracellular region, respectively. We also show that TRAF1 and TRAF3 have opposite effects to TRAF2 on NF-κB and JNK activation by TNFR2. Moreover, we show that TNFR2 is able to induce JNK activation in a TRAF2-independent fashion. This new receptor activity relies on a sequence located in the conserved module III. This region is also responsible for the ability of TNFR2 to induce TRAF2 degradation, thus emphasizing the role of conserved module III (amino acids 338–379) on receptor signaling and regulation. We show that only TNFR2 can induce TRAF2 degradation while TRAF1 or TRAF3 is not subjected to this regulatory mechanism and that TRAF1, but not TRAF3, is able to inhibit TRAF2 degradation.

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1. Introduction

TNF has been established as a main player in the immune processes of the organism [1,2]. In the last few years, the role of the inflammatory reactions on cancer development [3] has highlighted the role of TNF both in tumor development [4–7] and inhibition [8,9]. The study of TNF structure and functions led to the identification of one of the main families of cytokines, the so-called TNF superfamily which exerts its biological effects through its interaction with transmembrane proteins which constitute the TNFR superfamily of receptors [10–12]. TNFRs trigger their biological responses upon binding of different adaptor proteins and may lead to final responses as diverse as cell proliferation or cell death. The TRAF proteins were initially identified because of their interaction with different TNFRs, regulating them and acting as transducers for different biological pathways [13]. In mammals, the TRAF family is constituted of 7 different members. All these proteins contain the TRAF domain, involved in their interaction with the TNFRs and with other TRAF proteins [14,15]. Except for TRAF1, all TRAF proteins contain a RING domain in their N-terminal region. The deletion of this domain in TRAF2, TRAF5 or TRAF6 leads to the generation of dominant negative mutants, indicating that it is essential for the biological activity of the protein [16,17]. More recently, several studies have suggested an E3 ligase activity for this RING thus pointing to a role in ubiquitination involved in the regulation of TNFR signaling [18, 19]. Binding of TRAF proteins to TNFRs occurs through highly defined motifs present on the receptors. Two sequence motifs have been suggested to be implicated in TRAF proteins binding. One is constituted by the sequence (P/S/A/T)x(Q/E)E and the other one is represented by the sequence PxQxxD [14].

Both TNF receptors TNFR1 and TNFR2 are transmembrane proteins with high similarity in their extracellular regions although they differ widely in their intracellular domains. TNFR1 contains in its intracellular region a death domain while TNFR2 does not contain a death domain. Instead, it has two different regions implicated in the binding of TRAF proteins [20,21]. Although TNFR1 signaling has been widely characterized and reviewed [22], TNFR2 activity is less well understood. Most of the biological activities triggered by TNFR2 depend on its interaction

Abbreviations: AIP1, ASK1 interacting protein; AP-1, activating protein 1; BKO, binding knock out; CRMA, cytokine response modifier A; cIAP, cellular inhibitor of apoptosis; DMEM, Dulbecco modified Eagle's medium; FLAG, FLAG peptide; HA, hemagglutinin; HEK, human embryonic kidney; IKK, IκB kinase; IκB, inhibitor of NF-κB; JNK, N-terminal Jun kinase; NF-κB, nuclear factor κB; PEI, polyethylenimine; RANK, receptor activator of NF-κB; TNF, tumor necrosis factor; TNFR, TNF receptor; TRADD, TNF receptor associated protein with death domain; TRAF, TNF receptor associated factor.

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with TRAF2 which leads to the activation of both NF-κB and AP-1 [23]. Here, we have focused on characterizing the interaction of TNFR2 with TRAF proteins and studied the functional consequences of this interaction. We show that TRAF1, TRAF2 and TRAF3 bind to the receptor through different regions of its intracellular region and that TRAF1 and TRAF3 binding have opposite effects to that of TRAF2 binding on NF-κB and JNK activation. Moreover, we show for the first time that TNFR2 is able to induce c-Jun phosphorylation in a TRAF2-independent fashion. This new receptor activity relies on a region of the receptor in which our studies have also localized the ability of TNFR2 to induce TRAF2 degradation [20], thus emphasizing the role of module III (amino acids 338–379) on receptor signaling and its regulation.

2. Materials and methods

2.1. Cell lines and transfection reagents

HEK293 cell line was obtained from the American Type Culture Collection (Rockville, MD) and cultured in minimal essential medium supplemented with 10% fetal bovine serum, 1% glutamine, 0.13% bicarbonate and antibiotics (100 U/ml penicillin, 50 mg/ml streptomycin). For transient transfections, HEK293 cells (2×10^5 cells/well on 6-well plates) were seeded and transfected with 4.25 μg PEI pH 7.5 with the indicated plasmids in each case in 200 μl of DMEM supplemented with 1% of non-essential amino acids following the manufacturer's instructions. Cells were harvested 36 h after transfection.

2.2. Plasmids and antibodies

Expression plasmid encoding human Myc-tagged TRAF2 as well as the plasmids encoding the adaptor proteins TRAF1 and TRAF3 have been described elsewhere [24]. Expression plasmids pCR3-FLAG encoding the adaptor proteins TRAF1, TRAF2, TRAF3 and TRAF6 as well as the plasmid pCRK-myc-TRAF5 were kindly provided by Dr. Bryant G. Darnay, from the M.D. Anderson Cancer Center, Houston, TX. The NF- B reporter construct pNF- B-luc encodes the luciferase protein from firefly (*Photinus pyralis*) under the control of a promoter with several B elements. It was a generous gift from Dr. David S. Ucker, from the University of Illinois, Chicago. The pRL-TK plasmid (Promega) encodes the luciferase protein from *Renilla reniformis* under the control of the thymidine kinase promoter from HSV-TK herpes virus. The HA-JNK encodes the JNK protein tagged with the HA antigen and was a generous gift from Dr. Pilar de la Peña, from the University of Oviedo. Expression plasmids encoding human FLAG-tagged TNFR2 (pCMV1-FLAG-TNFR2) and human TNFR1 (pCDNA3-TNFR1) were a gift from B.B. Aggarwal (M.D. Anderson Cancer Center, Houston, Texas, USA). Expression plasmids for the GST fusion protein pGEX-KG and those encoding the intracellular region of RANK and TNFR2 receptors (pGEX-KG-RANK and pGEX-KG-TNFR2 respectively) were also kindly provided by Dr. Bryant G. Darnay, from the M.D. Anderson Cancer Center, Houston, TX. Unless otherwise indicated, TNFR2 constructs and GST-TNFR2 deletions used in this work were generated by PCR using standard methods and the primers indicated in Table SI. To generate point mutations by PCR mutagenesis, the Strategene Quick Change Site-Directed Mutagenesis Kit was used together with the primers indicated in Table SI. The sequences of all plasmids generated in this work were verified by automated DNA sequencing. Primary antibodies against TRAF1 (G-20), TRAF2 (C-20), TRAF3 (H-20) and TNFR1 (H-5) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and anti-FLAG peptide (F3165) and anti-β-actin (A5441) from Sigma. Anti-HA antibody was purchased from Roche and anti-c-Jun-phospho-Ser73 antibody was obtained from Cell Signaling. Secondary antibodies anti-rabbit IgG and anti-mouse IgG tagged with fluorophores were purchased from LI-COR Biosciences.

2.3. Western-blot analysis

Proteins were separated by SDS-PAGE, electroblotted onto PVDF membranes (Immobilone-FL, Millipore), blocked for 1 h in 5% non-fat milk and incubated with the indicated primary antibodies (at 1:5000 dilution in TBS-0.1% Tween) and the appropriate secondary antibody (at 1:15,000 dilution in 5% non-fat milk in TBS-0.1% Tween). Finally, the membranes were scanned with the Odyssey® Infrared Imaging System (LI-COR Biosciences).

2.4. Co-immunoprecipitacion assay

HEK293 cells were cotransfected with 1 μg of the plasmids indicated in each case together with 0.3 μg of the plasmids encoding the TRAF protein of interest. Twenty-four hours after transfection, cells were harvested and lysed in 200 μl Lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM DTT, 2 mM EDTA, 1% Triton X-100, 1 μg/μl leupeptin, 1 μg/μl aprotinin, 2 mM sodium orthovanadate). Two 20 μl fractions were collected as inputs for the TRAF protein and for the receptor as expression controls. The remaining 160 μl fraction was incubated with 1 μg anti-FLAG antibody at 4 °C in rotation for one hour and then with 20 μl protein G-Sepharose beads in rotation at 4 °C overnight. After three washes with lysis buffer and an additional one with low salt buffer (20 mM Tris-HCl pH 8.0, 25 mM NaCl, 1 mM DTT, 1 μg/μl leupeptin, 1 μg/μl aprotinine, 2 mM sodium orthovanadate), the immunoprecipitate was resuspended in SDS-sample buffer and subjected, together with inputs, to SDS-PAGE and Western blot analysis was performed with anti-TRAF1, anti-TRAF2 or anti-TRAF3 and anti-FLAG (anti-receptor) antibodies.

2.5. GST-TNFR2 fusion protein affinity binding assay

Equivalent amounts of each GST-TNFR2 fusion protein attached to 20 ml glutathione-agarose beads were mixed with lysates from HEK293 cells transiently transfected with the plasmid encoding the TRAF protein of interest in binding buffer (20 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% Triton X-100, 1 mM DTT, 1 mM sodium orthovanadate, 2 μg/ml aprotinine, 2 μg/ml leupeptin, 25 mM β-glicerophosphate) and allowed to rotate for 1 h at 4 °C. The beads were collected by centrifugation, washed three times in binding buffer and then washed once in low-salt buffer (20 mM HEPES pH 7.4, 25 mM NaCl, 1 mM DTT). Bound proteins were eluted with addition of SDS sample buffer and boiled. The eluted proteins were subjected to 7.5% SDS-PAGE and Western blot analysis was performed with either anti-Myc (TRAF5) or anti-FLAG (TRAF1, TRAF2, TRAF3 and TRAF6).

2.6. NF-κB activation

NF-κB activity was determined by an analysis of luciferase activity. HEK293 cells were transfected with 0.2 μg pNF-κB-luc, 0.05 μg pRL-TK and with the amounts of the plasmids of interest indicated in each case. Measurements of both luciferases were determined with the Dual-LuciferaseTM Reporter Assay System kit (Promega) following the manufacturer's instructions. On the graphic representations of the data the control condition (cells transfected with the pCMV1-FLAG vector alone) is considered the basal activation that obtained with. In all other conditions, the data are represented as activation fold over the control condition.

2.7. c-Jun phosphorylation

HEK293 cells were transfected with 1 μg of pHA-JNK and with the plasmids of interest indicated in each case. After 36 h, cells were harvested and resuspended in 200 μl of lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glicerophosphate, 1 mM sodium orthovanadate,

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