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Casein kinase 2-interacting protein-1, an inflammatory signaling molecule interferes with TNF reverse signaling in human model cells

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ABSTRACT

When transmembrane form of tumor necrosis factor (mTNF) interacts with its cognate receptors or agonistic antibodies signaling pathways are activated in the ligand expressing cells. This "reverse signaling" appears a fine-tuning control mechanism in the immune response. Despite a clinical relevance key molecules of TNF reverse signaling and their functions remain elusive. We examined the role of CKIP-1, an interacting partner of the N terminal fragment of mTNF in inflammation and TNF reverse signaling. We found that CKIP-1 expression was elevated upon LPS challenge in THP-1 human monocyte model cells. Overexpression of CKIP-1 triggered classical activation of THP-1 cells and transactivated the human TNF promoter when co-expressed with c-Jun in the HEK293 model system. TNF reverse signaling induced a massive translocation of CKIP-1 from the plasma membrane to intracellular compartments in THP-1 cells. Expression of the N terminal fragment of mTNF in HEK293 cells resembled the effects of TNF reverse signaling with respect to relocalization of CKIP-1. In parallel with the translocation, CKIP-1-triggered activation of THP-1 cells was antagonized by TNF reverse signaling. Similarly, the presence of the N terminal fragment of mTNF inhibited CKIP-1 mediated TNF promoter activation in HEK293 cells. Both TNF reverse signaling in THP-1 cells and expression of the N terminal fragment of mTNF in HEK293 cells were found to induce apoptosis that could be prevented by overexpression of CKIP-1. Our findings demonstrate that CKIP-1 activates pro-inflammatory pathways and interferes with TNF reverse signaling induced apoptosis in human model cells.

1. Introduction

Members of the TNF and TNF receptor superfamilies are transmembrane proteins, but many of these have soluble forms liberated by TACE (TNF-alpha converting enzyme) [1]. When TNF superfamily ligands and their cognate receptors interact, signaling pathways can be activated in both ligand and receptor bearing cells, leading to activation, differentiation or apoptosis. This bidirectional communication has been reported for a number of TNF superfamily members [2–9] and considered as a fine-tuning mechanism during the immune response. We and others reported receptor-like properties of the transmembrane TNF (mTNF), the first described member of the superfamily [10–14]. Soluble ectodomains of receptor molecule (*e.g.* Etanercept) or anti-TNF antibodies (*e.g.* Infliximab, Adalimumab) can also elicit reverse signaling in mTNF expressing cells [15,16]. Therapeutic application of these agents in chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease [17] revealed effects of TNF reverse signaling on different immune cells mostly depending on the cell type. Infliximab treatment induced apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. In monocytes from patients with rheumatoid arthritis, signaling through mTNF attenuated pathologic interleukin-1 beta production and corrected deficient apoptosis [18]. Apoptosis induction through TNF reverse signaling has been reported *in vitro* upon treatment either with TNF antibodies [15] or with soluble TNFR [19]. Interestingly, TNF reverse signaling attenuated the proliferative potential of T helper cells,

Abbreviations: ACP, actin capping protein; ATM, ataxia telangiectasia mutated; CKIP-1, casein kinase 2-interacting protein-1; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; EGFP/ECFP/EYFP, enhanced green/cyan/yellow fluorescent protein; HBSS, Hank's buffered salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; hTIP, human TNF intracellular domain interacting protein; mTNF, transmembrane tumor necrosis factor; NLS, nuclear localization signal; TNFNterm, N terminal fragment of TNF.

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meanwhile increased the cytotoxic potential of CD8+ T lymphocytes [20]. Moreover, TNF reverse signaling has been implicated in undesirable side-effects of anti-TNF therapies, where secondary infections were observed [21].

Despite the apparent clinical relevance the molecular basis of TNF reverse signaling is largely unknown. The first reports revealed protein kinase C and MAPK/ERK to be involved in TNF reverse signaling triggered LPS resistance in myeloid cells [14]. We reported earlier that the cytoplasmic domain of TNF is serine phosphorylated in mTNF expressing cells [10]. Moreover, interaction of mTNF with its soluble receptor triggers rapid dephosphorylation of mTNF and concomitant Ca^{2+} signaling [10]. The cytoplasmic serine residues of mTNF and phosphorylation of c-Jun N-terminal kinase have been proved essential for Infliximab-induced interleukin-10 production, apoptosis, and G0/G1 cell cycle arrest, where Infliximab treatment was shown to upregulate Bax, Bak, and p21 expression [15]. The MAPK pathway and caspases have been implicated in downstream signaling events of TNF reverse signaling [14], whereas other possible participating molecules remained elusive.

Identifying signaling molecules recruited to the mTNF molecule and investigating their role may help to unravel molecular details of TNF reverse signaling. A novel protein that interacts with the intracellular domain of mTNF has been identified (hTIP, human TNF intracellular domain interacting protein [gi:9622148]) and proved to be identical to human casein kinase 2-interacting protein-1 (CKIP-1) [22]. CKIP-1 has been found to interact with a series of proteins involved in cellular functions like differentiation, cell motility and cell death [23,24]. Since CKIP-1 has been identified as an interacting partner of the pro-inflammatory mTNF, we aimed at elucidating the function of CKIP-1 in inflammation and in TNF reverse signaling. Here we show novel biological properties of CKIP-1 that was found to activate the inflammatory response and to interfere with reverse signaling elicited by anti-TNF in human THP-1 cells.

2. Materials and methods

2.1. Cell culture

HEK293 cells (ATCC, Manassas, VA) and their derivatives were grown in a (1:1) mixture of Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) and Ham's F-12 nutrient medium (Sigma) supplemented with 10% low endotoxin FCS (Sigma) and 2 mM L-glutamine (Sigma). THP-1 cells were maintained in Opti-MEM (Invitrogen) with 2% FCS. For activation of THP-1 monocytes cells were challenged by a medium containing 10–1000 ng/ml LPS (Sigma), 50 μg/ml Infliximab (Remicade; Centocor B.V., The Netherlands), 150 μg/ml hIgG (Human gamma globulin; Human Bioplazma Kft., Hungary) or 5 μM etoposide (Sigma). Adherent THP-1 cells were generated by priming the suspension cells with 25 ng/ml PMA (Sigma) for 72 h. Adherent THP-1 cells were treated with $F(ab)_2$ fragment of Infliximab, generated by using $F(ab)_2$ Preparation Kit (Thermo Scientific, Rockford, IL).

2.2. Expression vectors

The human CKIP-1 coding sequence in pQBI-25 vector was kindly provided by Dr. Chie Kohchi (Hiroshima University, Japan). The coding sequence was cloned into pBluescript-SK+ (Stratagene, Santa Clara, CA) and into mammalian expression vectors pcDNA3 (Invitrogen), pEGFPC1, pECFPC1 (Clontech, Mountain View, CA) and p3xFlag-Myc-CMV-26 (Sigma). The 252 bp N terminal fragment of mTNF (TNFNterm) was fused to GST by cloning the cDNA into pGEX-4T1 (Amersham Biosciences, Germany) and was fused to EYFP or to mCherry by cloning into pEYFPN2 or pmCherryN2 (Clontech), respectively. The Path Detect Trans Reporting System (pFA2-c-Jun, pFR-Luc, pFc-MEKK, pFC-dbd) was purchased from Stratagene. The mammalian expression plasmid for c-Jun (pcDNA3-c-Jun) was kindly provided by Dr. Imre Kacskovics (Eötvös L. University, Hungary). Enzymes used for *in vitro* recombinant DNA techniques were purchased from Fermentas and New England Biolabs.

2.3. Transient transfections and reporter gene assays

Construct pGL3-luc-TNFprom (pGL3-luc from Promega) in which the luciferase gene was controlled by the TNF-alpha promoter (−801 to +1) was introduced into HEK293 cells by Lipofectamine-2000 (Invitrogen). HEK293 stable clones were isolated after G418 (Sigma) selection. Stable clones (2×10^5 cells) containing the luciferase reporter construct were transiently co-transfected in 24-well plates with 100 ng of expression vectors for CKIP-1 and c-Jun using JetPEI (Poly Transfections, France) according to the manufacturer's protocol. Total amount of DNA transfected was kept constant by adding empty vector. In luciferase assays cells were harvested 36 h after transfection and total cell extracts were prepared. Briefly, cells were harvested in 50 μl of lysis buffer (Promega, Madison, WI). Crude cell lysates were cleared by centrifugation, luciferase activity of 20 μl cell extracts was measured after injection of 20 μl Bright and Glow substrate (Promega) in a Luminoscan Ascent luminometer (Labsystems, Oy, Finland). To analyze the effect of CKIP-1 on the transcriptional activity of c-Jun the PathDetect™ Trans-Reporting System was used (Stratagene). Transient transfections were performed using JetPEI. Briefly, 10^5 HEK293 cells were seeded in 24-well plates 16 h prior to transfection. Each point was co-transfected with 500 ng of pFR-Luc, 100 ng of pFA2-c-Jun and 100 ng of the positive control vector pFc-MEKK or 100 ng of pQBI-hCKIP-1. Parallel experiments were performed by co-transfecting the empty vector pQBI-25. Cells were grown in serum free medium for 6 h after transfection, then 10% FCS containing culture medium was added and the cells were incubated for 24–36 h. Total cell extracts were prepared for luciferase assays as described above. For flow cytometry and fluorescence microscopy experiments, THP-1 and HEK293 cells were transiently transfected by an Amaxa Nucleofector device (Amaxa, Germany) following the manufacturer's protocols and using Amaxa® Cell Line Nucleofector® Kit V (Lonza) or Lipofectamine-2000 (Invitrogen), respectively.

2.4. Protein expression, purification and GST-pull down assay

GST fusion proteins were purified from *E. coli* BL21 (DE3) lysates using 50% slurry of glutathione-Sepharose beads (Amersham Pharmacia Biotech). pBS-hCKIP-1 (0.5 μg) was transcribed and translated *in vitro* using the TNT coupled reticulocyte system (Promega) in a total volume of 25 μl according to the manufacturer's protocol. In each of the pull-down experiments 10 μl of *in vitro* translated ^{35}S-labeled protein was diluted in 100 μl binding buffer (20 mM HEPES (pH 7.3), 100 mM KCl, 0.2% Nonidet P-40, 1 mM PMSF). About 2 μg of fusion protein or GST immobilized on glutathione-Sepharose beads were added, and the interactions were allowed to proceed by rotation at room temperature for 30 min. Beads were washed five times with binding buffer. Proteins bound were separated on a 12.5% SDS-polyacrylamide gel. ^{35}S-labeled proteins were detected by autoradiography.

2.5. Cell lysis and immunoprecipitation

24 h post-transfection HEK293 cells were washed in ice-cold PBS, scraped in lysis buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 1%