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Identification of RNF114 as a novel positive regulatory protein for T cell activation

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ABSTRACT

RNF114 [RING (really interesting new gene) finger protein 114] has been shown to be a novel psoriasis susceptibility gene, with a putative role in the regulation of immune responses, though the underlying mechanism was not fully identified. In the present study, to investigate whether RNF114 is involved in T cell activation, a series of fluorescence activated cell sortings (FACS) were performed. The analysis confirmed that RNF114 over-expression had a promotion effect on T cell activation with an average 43.97% increment and the upregulatory roles showed a dose-dependent effect with 18.44% increment. Interestingly, the two C2H2 domains were shown to play important but opposite roles in T cell activation. The deficiency of upstream C2H2 domain increased the efficiency of T cell activation by 12.81%, while the downstream C2H2 domain alone promoted it with an average level 25.12% higher than intact RNF114 protein. Combined with tandem affinity purification (TAP) and mass spectrometry, our investigations found 23 RNF114-interacting proteins that have distinct physiological roles in transcription, translation, DNA repair and signaling pathways. These findings, including recognition of RNF114 as a positive regulatory protein and identification of its interacting proteins, widen the understanding for investigating functions of RNF114 involved in T cell activation.

Introduction

T cell activation is essential for the initiation of an adaptive immune response which is highly regulated by numerous regulators. Identification of these regulators and their roles is important for the understanding of immune mechanism (Jones and Thompson 2007; Singer and Koretzky 2002).

Recently, RNF125 (alias T cell RING protein in activation 1, TRAC-1), a RING domain protein predominantly expressed in lymphoid cells, was identified as a novel and unique positive regulator of T cell activation among ubiquitin ligases in a functional screen for T cell regulators (Chu et al. 2003; Giannini et al. 2008). Homologous protein BLAST revealed that this protein belongs to a subfamily of ubiquitin ligases with zinc fingers, including RNF114 (alias ZNF313), RNF138 (alias NLK-associated RING finger protein, NARF) and RNF166 (Giannini et al. 2008; Capon et al. 2008). The members of this subfamily comprise five highly conserved domains, an amino-terminal C3HC4 (Cys3-His-Cys4) RING finger domain, a central C2HC (Cys2-His-Cys) and two C2H2 (Cys2-His2)-type zinc fingers, as well as a carboxy-terminal UIM (ubiquitin interacting motif) (Giannini et al. 2008). The domain similarity among these family members suggests that these proteins may have similar functions in cell physiology.

Among them, RNF125 has been identified as a positive-mediator of T cell activation, RNF138 has been shown as an inhibitor of Wnt signaling and the data found by our group has pointed RNF166 to play a potential role involved in the positive regulation of immune responses (Chu et al. 2003; Yang et al. 2013; Yamada et al. 2006).

In previous reports, RNF114 which was initially identified by our group has been described as a psoriasis susceptibility gene by genome-wide association studies (GWAS) (Capon et al. 2008; Ma et al. 2003). RNF114 over-expression may enhance NF-κb and IRF3 reporter activity and increase type I and type III interferon (IFN)

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mRNA levels which indicate that RNF114 regulates a positive feedback loop that enhances dsRNA induced production of type I IFN in the regulation of RIG-I/MDA5 signaling pathway (Bijlmakers et al. 2011; Onoufriadis et al. 2012). However, the roles of RNF114 are not fully defined.

In the present study, we provide new insights of RNF114 in immune response events. Our experiments show that RNF114 is a novel positive regulator of T cell activation and suggest that C2H2 domains are involved in the function regulation of RNF114. 23 RNF114-interacting proteins were identified with TAP (tandem affinity purification) combined with mass spectrometry technology. These TAP results suggest that RNF114 may contribute to a number of physiological responses, including transcription, translation, DNA repair and replication, signaling pathways and ubiquitination, metabolism and mitochondrial fusion.

Materials and methods

Antibodies and reagents

The antibodies and reagents used in the present study include Pan T cell Isolation Kit II human (Miltenyi), InterPlay Mammalian TAP System (Stratagene), anti-RNF114 mAbs (monoclonal antibodies) (Abcam), anti β-actin rabbit pAbs (polyclonal antibodies) (AVIVA), Dynabeads CD3/CD28 T Cell Expander (Invitrogen), APC (allophycocyanin) conjugated anti-CD69 mAbs and PE (phycoerythrin) conjugated anti-CD3e mAbs (eBioscience), horseradish-peroxidase-conjugated goat anti-rabbit and rabbit anti-mouse antibodies (Zhongshan Goldenbridge).

cDNAs and constructs

RNF114 cDNA was amplified from cDNA of human primary T cells using primers based on the RNF114 mRNA sequence (GenBank accession number NM_018683). The domain truncated mutants RNF114-Δ84, ΔN76-228, ΔN76-129, delN76-129, ΔN121-172, delN121-172, ΔN165-207, delN165-207, ΔN200-228 and ΔN207 were generated by site-directed mutagenesis. For T cell activation experiments, RNF114 and the mutant cDNAs were subcloned into pEGFP-N1 (Clontech) to express proteins with a C-terminal GFP (green fluorescent protein) tag. For tandem affinity purification and quantitative mass spectrometry experiments, RNF114 cDNA was subcloned into pNTAP-A (Stratagene) to express N-terminal CBP (Calmodulin binding peptides) and SBP (Streptavidin binding peptides) fusion proteins. All RNF114 constructs were verified by sequencing.

Cell preparation and transfections

Jurkat T cells (Clone E6-1, ATCC) were maintained in RPMI 1640 medium (Gibco) (Giannini et al. 2008). Primary human T cells were enriched from human peripheral blood mononuclear cells (PBMCs) by the depletion of indirectly magnetically labeled non T cells using Pan T cell Isolation Kit II (Miltenyi) and were maintained in RPMI 1640 medium (Gibco) (Giannini et al. 2008). Normally, more than 95% pure T cell populations were isolated, measured by flow cytometry analysis staining with anti-CD3e-PE (phycoerythrin). The human peripheral blood samples were obtained from healthy adult volunteers in West China First University Hospital. The study was approved by the committee of Ethics of Biological and Medical Research, Sichuan University, and all the participants gave written informed consent for scientific research. For transfection, 10^7 Jurkat T cells and 5×10^6 primary T cells were electroporated in 250 μl of OPTI-MEM I Reduced Serum medium (Gibco), using 0.2 cm cuvettes and Gene Pulser Xcell II Electroporation System (Bio-Rad) at 180 V and 1000 μF in the presence of 20 μg of DNA.

T cell activation and FACS analysis

T cells were stimulated by adding 2 μl Dynabeads Human T-Activator CD3/CD28 to obtain a bead-to-cell ratio of 1:1. At 16 h post-stimulation in a humidified CO_2 incubator at 37 °C, cells were stained with APC-conjugated anti-human CD69 mAb in 2% FCS in PBS on ice for 30 min avoiding light. Subsequently, cells were fixed in 4% paraformaldehyde and counted by flow cytometry using FACScalibur (Becton Dickinson) gated on healthy cells based on forward and side scatter properties. Instrument settings were optimized by transfected but unstained, GFP^+ and APC-CD69-stained GFP^- cells. Analysis of FACS plots was carried out by FCS Express Version IV software (De Novo) and CellQuest (Becton Dickinson).

Membrane flotation assays, immunofluorescence and western blot

To investigate whether RNF114 is membrane-associated or soluble protein in T cell, membrane flotation assays were performed as described previously (Giannini et al. 2008). For the investigation of RNF114 subcellular localization, Jurkat T cells were spreaded onto 13 mm glass coverslips 16–24 h after transfection and fixed with 4% paraformaldehyde for 30 min and observed using FV1000 confocal microscope (Olympus).

Proteins were extracted in universal protein extraction buffer (Bioteke) containing 5 μl/ml EDTA-free protease inhibitor Cocktail (Roche). For the detection of proteins, protein samples were denatured in SDS sample buffer for 5 min at 95 °C, and loaded on 10% SDS polyacrylamide gels. SDS-PAGE and immunoblotting were performed with standard protocol.

Tandem affinity purification and mass spectrometry analysis

For the detection of the potential interacting proteins of RNF114, Jurkat T cells were transfected with RNF114 constructs cloned into pNTAP-A plasmid. Transformants were selected by growth in medium contained G418, and RNF114 protein complexes of interest were purified using affinity purification tags (SBP and CBP) with subsequent mass spectrometry identification of unknown protein complex components.

Statistical and bioinformatical analysis

All experiments were repeated at least three times unless stated otherwise. The statistical analysis were performed with GraphPad Prism 5 software (GraphPad Software, Inc), using *t* test unless stated otherwise. The mass spectrometry data were searched and analyzed against the human IPI database at the Universal Protein Resource, UniProt (http://www.uniprot.org).

Results

RNF114 is a soluble protein endogenously expressed in T cell

Previous studies carried out by our group and others have demonstrated that RNF114 transcript can be detected in a wide range of cell types, with the highest levels found in testis (Capon et al. 2008; Ma et al. 2003; Li et al. 2007). By using western blot analysis, we confirmed that RNF114 protein endogenously expressed both in primary and Jurkat T cells (Fig. 1A). To explore the subcellular localization of RNF114 in T cell, immunofluorescence assay using confocal microscopy was performed. The results showed that RNF114 protein can be diffusely observed both in