ATAR, a Novel Tumor Necrosis Factor Receptor Family Member, Signals through TRAF2 and TRAF5*

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Members of tumor necrosis factor receptor (TNFR) family signal largely through interactions with death domain proteins and TRAF proteins. Here we report the identification of a novel TNFR family member called ATAR. Human and mouse ATAR contain 283 and 276 amino acids, respectively, making them the shortest known members of the TNFR superfamily. The receptor is expressed mainly in spleen, thymus, bone marrow, lung, and small intestine. The intracellular domains of human and mouse ATAR share only 25% identity, yet both interact with TRAF5 and TRAF2. This TRAF interaction domain resides at the C-terminal 20 amino acids. Like most other TRAF-interacting receptors, overexpression of ATAR activates the transcription factor NF-κB. Co-expression of ATAR with TRAF5, but not TRAF2, results in synergistic activation of NF-κB, suggesting potentially different roles for TRAF2 and TRAF5 in post-receptor signaling.

The TNFR superfamily includes, among others, TNFR1, TNFR2, Fas, NGFR, CD40, and CD30 (1). Receptors within this group share a similar extracellular domain architecture of multiple cysteine-rich repeats, each containing about 40 amino acids, with six cysteines. However, no significant homology is found within the intracellular domains with the exception of the death domain shared by TNFR1, Fas, and Ws-1 (DR3) (2–5). Post-receptor signaling mechanisms utilized by the TNFR superfamily have recently begun to emerge with the discovery of two distinct classes of receptor-associated proteins.

The death domain proteins TRADD, RIP, and FADD interact with the death domain of TNFR1 or Fas following ligand-mediated receptor aggregation (6–9). The N-terminal death effector domain of FADD then recruits Caspase-8 (Flice/MACH), an ICE-like cysteine protease, to the receptor complex (6–8). This process activates the apoptotic protease cascade and results in rapid onset of apoptosis.

The second group of signaling molecules, the TRAF proteins, directly interact with TNFR2, CD40, CD30, and lymphotixin-β receptor (9–12). No obvious motifs are shared by the cytoplasmic regions of these TRAF-binding receptors except for a few conserved amino acids. Six TRAF proteins have been reported, each containing a highly conserved TRAF domain at the C terminus and, with the exception of TRAF1, a lesser conserved N-terminal RING finger and zinc finger motif (9–11, 13–15). Specific receptor recognition is mediated through the TRAF domain. Biological activities and post-receptor signaling mechanisms of the TRAF proteins are still obscure. TRAF2, TRAF5, and TRAF6, when overexpressed, activate NF-κB (11, 13, 16). TRAF2 is also required for TNF-induced JNK activation (17). The N-terminal RING finger motif is necessary for both of these activities.

In the course of Amgen EST project, we discovered a novel TNFR-related protein we have named ATAR (another TRAF-associating receptor). Expression of ATAR is highly tissue-specific. To explore its biological function, we applied the yeast two-hybrid system to screen for signaling proteins that associate with the intracellular domain of ATAR. We found that ATAR interacts with both TRAF2 and TRAF5. Like most other TRAF-binding TNFR family members, ATAR also mediates NF-κB activation.

MATERIALS AND METHODS

Reagents—293 cells (ATCC) were maintained in high glucose DMEM containing 10% fetal calf serum, 100 μg/ml penicillin G, and 100 μg/ml streptomycin. Constructs containing a GAL4 DNA binding domain fused with hATAR(224–283), hNTR(224–263), hTAR(264–283), or mATAR(221–276) were generated by cloning PCR fragments into pGBl9 (CLONTECH). C-terminal point mutants of ATAR were generated by PCR using primers containing the corresponding nucleotide substitutions. An ATAR expression vector was constructed by cloning a 1.4-kilobase pair SalI-EcoRI fragment into pCDNA3.1(−) under transcriptional control of the CMV promoter. FLAG-TRAF5, FLAG-TRAF3(205–558), and FLAG-TRAF2 expression vectors were generated by cloning cDNA fragments in-frame with DNA encoding an N-terminal FLAG epitope. Constructs containing TRAF1 or TRAF3 fused with GAL4 DNA-binding domain and the NF-κB reporter construct were described previously (18).

Identification, cDNA Cloning, and Rnase Protection—Mouse ATAR cDNA was identified during the Amgen EST Project as described (19). Full-length mouse and human clones were isolated by screening mouse intestinal and human bone marrow cDNA libraries, respectively, using mouse ATAR cDNA as a probe as described (19). RNase protection assay was performed with a commercially available kit according to the manufacturer’s recommendations (Ambion).

Yeast Two-hybrid Screening—DNA encoding the intracellular domain (amino acids 224–283) of ATAR was cloned in-frame with the GAL4 DNA-binding domain in pGPT9 vector, and the resulting plasmid was transfected into 293-EBNA-1 cells using lipofectamine (Life Technologies, Inc.), and transfected cells were selected with 100 μg/ml hygromycin. Drug resistant clones were pooled and cultured in serum-free medium for 72 h. ATARFc fusion protein was purified from the conditioned medium by protein G affinity chromatography as described.

Antibody Generation—A PCR fragment encoding the ATAR extracellular domain was fused in frame upstream of human IgG1 Fc in a modified pCPE4 vector (Invitrogen). The resultant ATARFc/pCPE4 plasmid was transfected into 293-EBNA-1 cells using lipofectamine (Life Technologies, Inc.), and transfected cells were selected from 100 μg/ml hygromycin. Drug resistant clones were pooled and cultured in serum-free medium for 72 h. ATARFc fusion protein was purified from the conditioned medium by protein G affinity chromatography as described.

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1 The abbreviations used are: TNFR, tumor necrosis factor receptor; PCR, polymerase chain reaction.
scribed (20). The fusion protein was then used as antigen to raise rabbit anti-ATAR antiserum (Babco).

Transfection and Reporter Assay—Approximately 3 × 10⁵ 293 cells/well were seeded on 6-well plates. The next day, cells were transfected by the calcium phosphate precipitation method. After incubation for another 24 h, cells lysates were prepared and tested for luciferase activity using the Enhanced Luciferase Assay kit (Analytical Luminescence Laboratory).

β-Galactosidase activity was measured with Galacto-Light (Tropix).

Co-immunoprecipitation and Western Analysis—Transfections were performed in 6-well plates as described above. 24 h after transfection, cells were lysed with 500 μl of E1A buffer (50 mM Hepes [pH 7.6], 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA). Lysates were then incubated with 5 μl of preimmune serum or antisera against ATAR extracellular domain at 4 °C for 1 h and then mixed with 20 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia Biotech Inc.) and incubated for another hour. After six washes with 1 ml of E1A buffer, the precipitates were fractionated into 12.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Schleicher & Schuell). Western blot analysis was performed with an anti-FLAG monoclonal antibody (Kodak) and horseradish peroxidase-coupled rabbit anti-mouse immunoglobulin using enhanced chemiluminescence according to the protocol of the manufacturer (Amersham Corp.).

RESULTS

A murine cDNA sequence that appeared to encode a portion of a novel TNFR family member was identified during the Amgen EST program. A full-length cDNA isolated from a mouse intestinal cDNA library encodes a 276-amino acid pro-
tein we called ATAR that has a signal peptide and a single transmembrane region (Fig. 1A). The extracellular domain contains three cysteine-rich repeats and is most homologous to TNFR2. The receptor has a short cytoplasmic tail of only 46 amino acids. During the screen, we also isolated different spliced products that result in deletions within the extracellular domain or generation of soluble receptor forms (Fig. 1B). However, none of the soluble forms comprise complete cysteine repeats, suggesting they might be defective in ligand recognition. The human counterpart of the receptor was subsequently isolated from a bone marrow cDNA library. Amino acid comparisons demonstrated that the mouse and human homologs share 51 and 25% identity within their extracellular and intracellular domains, respectively.

Tissue distribution of mouse ATAR was examined in an RNase protection assay (Fig. 2). Among the tissues examined, ATAR mRNA was highly expressed in spleen, thymus, bone marrow, lung, and small intestine. Very low levels of expression were detected in brain, skeletal muscle, and stomach. Similar tissue distribution patterns were observed for human ATAR mRNA as determined by Northern blot analysis (data not shown).

To explore the biological function of ATAR, we used yeast two-hybrid screening to search for signaling proteins that interact with the ATAR intracellular domain. Human B lymphocyte and mouse lymph node libraries were screened with bait containing the GAL4 DNA-binding domain fused to the human ATAR intracellular domain. A total of 40 million transformants were screened and positive clones obtained encoded TRAF5 or TRAF2. All of these TRAF5 and TRAF2 clones contained a complete C-terminal TRAF domain, consistent with previous reports that this domain mediates receptor interaction. We also examined the interaction of ATAR with TRAF1 and TRAF3 by yeast two-hybrid interaction assays. No interaction of ATAR with TRAF1 or TRAF3 was detected in this assay (Fig. 3A).

Because human and mouse ATAR are poorly conserved within their intracellular domains, we tested the ability of mouse ATAR to interact with TRAF proteins. Like its human homolog, mouse ATAR interacted with TRAF2 and TRAF5 but not TRAF1 or TRAF3 (Fig. 3A). Sequence alignment revealed a stretch of nine conserved amino acids (residues 261–269 in mouse and 264–272 in human) among otherwise largely unrelated intracellular domains (Fig. 1A), suggesting the importance of this short conserved region in TRAF recognition. To further localize the TRAF-interacting region, we fused residues 224–263 or residues 264–283 of the human ATAR intracellular domain to the GAL4 DNA-binding domain and tested their interaction with TRAF proteins. The GAL4DB-ATAR(264–283) fusion protein containing the conserved nine-amino acid stretch was able to bind both TRAF5 and TRAF2. Therefore, the C-terminal 20 amino acids of ATAR were sufficient for TRAF interaction. Interestingly, the last four of these nine amino acids are conserved in the TRAF-interacting region of CD40 (Fig. 3B). Double mutations of CD40 Glu253 and Thr254 to alanine abolished the TRAF interaction (21). Each of the four conserved amino acids of ATAR was substituted with alanine, and each point mutant was tested for its interaction with TRAF2 and TRAF5 (Fig. 3B). The glutamic acid to alanine substitution at position 271 abolished ATAR interaction with the TRAF proteins. However, the other point mutants interacted as well as the wild type receptor with TRAF2 and TRAF5.

We then examined the interaction of ATAR with TRAF proteins in mammalian cells. An expression vector that directs the synthesis of full-length ATAR was co-transfected with a FLAG-tagged TRAF2 or TRAF5 expression vector into human embryonic kidney 293 cells. Cell lysates from each transfection were immunoprecipitated with either preimmune serum or a polyclonal antibody against the extracellular domain of ATAR. Western analysis of the immunoprecipitates were performed with a monoclonal antibody against the FLAG epitope. Both Flag-TRAF2 and Flag-TRAF5 specifically co-precipitated with
ATAR (E271A), a point mutant that no longer binds with NF-κB. We also examined was co-expressed with TRAF2 (Fig. 5A). This NF-κB activation requires ATAR interaction with TRAF proteins, because expression of ATAR(E271A), the point mutant that no longer interacts with TRAF2 or TRAF5, failed to activate NF-κB (Fig. 5A).

Both TRAF2 and TRAF5, when overexpressed, activate NF-κB. This activity depends on their N-terminal RING finger and zinc finger motifs (11, 16). We generated a TRAF5(205–558) truncation mutant lacking the RING and zinc finger deletion mutant (data not shown). These dominant negative inhibitory effects suggest potential involvement of both TRAF5 and TRAF2 in ATAR-mediated NF-κB activation pathway.

To further examine the effects of TRAF5 and TRAF2 expression on ATAR-mediated NF-κB activation, we co-expressed ATAR with full-length TRAF5 or TRAF2 proteins in 293 cells. Interestingly, we found that co-expression of ATAR and TRAF5 resulted in a synergistic activation of NF-κB (Fig. 5C). On the contrary, no synergy was detected when ATAR was co-expressed with TRAF2 (Fig. 5C). We also examined ATAR(E271A), a point mutant that no longer binds with TRAF proteins. When co-expressed with TRAF5 or TRAF2, ATAR(E271A) did not affect NF-κB activation induced by TRAF5 or TRAF2 (data not shown). This specific synergistic activation by ATAR and TRAF5 suggests that TRAF5 plays a unique role in ATAR signaling.

**DISCUSSION**

We report here the identification of a novel TNFR family member. ATAR is the smallest reported member of this receptor superfamily so far. The human and mouse homologs are poorly conserved (45% identity) as compared with other members within the family. For example, TNFR1, TNFR2, Fas, and CD40 share 65, 64, 51, and 63% identity in the two species, respectively. This poor sequence conservation is especially evident in the region of the short cytoplasmic tail (25% identity). However, we argued against the possibility that they may represent distinct receptors based on two factors: 1) no other human counterparts were isolated during cDNA library screening with mouse ATAR, and 2) distinct receptors within this family share approximately 25% between each other.

Despite the limited homology, both human and mouse ATAR intracellular domains interact with the same TRAF proteins. This TRAF-binding activity resides in the C-terminal 20 amino acids of human ATAR, because GAL4-ATAR(264–283) is still able to interact with TRAF5. Interestingly, within this region is a stretch of nine amino acids well aligned with the corresponding region in the mouse receptor. Similar amino acid residues have been shown to be crucial in TRAF-recognition by TNFR2, CD40, and CD30. This finding should help shed light on the structural requirements of TRAF-receptor interactions.

Overexpression of ATAR in mammalian cells results in NF-κB activation. This activation depends on the interaction of ATAR with TRAF proteins, because a TRAF-binding deficient mutant (E271A) fails to activate NF-κB. In addition, we found that co-expression of ATAR with TRAF5 but not TRAF2 resulted in synergistic activation of NF-κB. These results suggest that TRAF5 contributes to ATAR-mediated NF-κB activation. However, it does not rule out involvement of TRAF2 in this process, because TRAF2 is ubiquitously expressed and might not be a limiting factor in 293 cells.

ATAR is identical to a recently reported protein (HVEM) that was identified as a receptor for mediating viral entry of herpes simplex virus-1 and -2 (22). It will be interesting to determine if this viral entry process activates ATAR and how cellular responses mediated by the receptor (e.g. NF-κB activation) affect the viral life cycle. It is noteworthy that latent infection membrane protein-1 (LMP1) of Epstein-Barr virus also interacts with TRAF proteins through its C-terminal cytoplasmic tail. Possible pathogenic roles of ATAR and TRAF proteins in herpes simplex virus infection await further study.

ATAR is mainly expressed in spleen, thymus, bone marrow, and small intestine, all tissues in which lymphoid cells are enriched. This expression pattern suggests that ATAR plays a role in development or regulation of the immune system. This notion is supported by the fact that ATAR mediates activation of NF-κB, which in turn activates a battery of genes crucial to immune regulation (23). Among the known TNF-like ligands tested, we did not detect interaction of ATAR with TNFα, lymphotoxin α and β, or CD40 ligand (data not shown). Identification of its cognate ligand will greatly improve our understanding of the physiological role of ATAR.

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