Identification of a Novel Activation-inducible Protein of the Tumor Necrosis Factor Receptor Superfamily and Its Ligand*

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EXPERIMENTAL PROCEDURES
cDNA Cloning—A data base containing more than 2 million ESTs obtained from over 750 different cDNA libraries was generated by Human Genome Sciences, Inc., using high throughput automated DNA sequence analysis of randomly selected human cDNA clones. A specific homology and motif search using the known amino acid sequence and motif of TNFR members against this data base revealed several ESTs with a translated sequence 35–55% homologous to that of the TNFR family. Several clones were identified from cDNA libraries of PHA-activated T cells, T helper cells, leucocytes, a healing abdomen wound, primary dendritic cells, and adipose tissue. A full-length AITR cDNA clone encoding an intact N-terminal signal peptide was obtained from a human activated T cell library and selected for further investigation. The complete cDNA sequence of both strands of this clone was determined, and its homology to TNFR members was confirmed. Similarly, TL6 (TNF ligand 6) was identified through a systematic comparison of sequence homology with TNF ligand family members. Partial TL6 sequences, which were 25% homologous to that of TNF ligand family members, were identified from endothelial, HUVEC (human umbilical vein endothelial cell), brain, and fetal liver cDNA libraries. A full-length cDNA clone was obtained from a human brain cDNA library.

Expression Vectors—Full-length and HA (hemagglutinin A epitope)-tagged AITR encoding the putative entire AITR protein (amino acids 26–234) were amplified by PCR using sense (5′-CTAGCTAGCTAGV...

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**Fig. 1. Sequence and expression analysis of AITR.** A, deduced amino acid sequence of AITR. The potential signal sequence is underlined. The putative glycosylation site is indicated as bold characters, and the transmembrane region is indicated in boxes. B, comparison of the amino acid sequence of AITR with murine GITR. Bold letter cs within three cysteine pseudorepeat motifs indicate conserved cysteine residues found in the extracellular domain of TNFR superfamily members. Conserved acidic amino acid clusters of the cytoplasmic domain are indicated in boxes. C, expression of AITR: Northern blot analysis in various tissues and cancer cell lines (left panels) and RT-PCR analysis in human PBMC (right panels). Northern blots of poly(A) RNA from human tissues and cancer cell lines were analyzed by hybridization to a 32P-labeled cDNA probe containing the entire AITR coding regions. For RT-PCR, 2.0 × 10^6 human PBMC were activated by treatment with different stimuli (dexamethasone (Dex), PMA/ionomycin (P/I), or αCD3/αCD28 mAbs) for 24 and 48 h. Total RNA was extracted using TRIzol. Treatment with different stimuli (dexamethasone, PMA/ionomycin, or anti-CD3/CD28 mAbs) for 24 and 48 h.

**Northern Blot and RT (Reverse Transcriptase)-PCR Analysis—**

For Northern analysis, cDNA probes were labeled with 32P using the Rediprime DNA labeling system (Amersham Pharmacia Biotech), and visualized using the enhanced chemiluminescence Western blot detection system (Amersham Pharmacia Biotech). Analysis of NF-κB by Reporter Assay—Approximately 0.5 × 10^6 HEK293 EBNA cells/well were seeded on 6-well plates. After 24 h, cells were transfected by the standard calcium phosphate precipitation method using various combinations of pcDNA3.1/CD5L-AITR plus pRK5 plasmids encoding TRAFs, dnTRAF2, NIK, or dnNIK. The total amount of plasmid was adjusted to 2.0 μg by adding empty vector. Twenty-four hours after transfection, cells were lysed in 200 μl of reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using 20 μl of cell extract. 5 μl of cell extract was used to assay β-galactosidase activity as an internal control, and luminescence values were normalized by individual β-galactosidase activity. Recombinant Protein Production and Purification—AITR-Fc fusion protein was used for ligand screening and cell-binding experiments. A fragment encoding the predicted extracellular domain of AITR (amino acids 26–139) was amplified using a sense primer flanked by an NheI site (5’-AGAGCCCAGCTCTGGGGCCTGCTGAGA and a primer flanked by a BglII site (5’-GAGAAATGCCTGGGCTCTGGGGGCCTGAGA-3’) and an antisense primer flanked by a BglII site (5’-GAGAAATGCCTGGGCTCTGGGGGCCTGAGA-3’) and an antisense primer flanked by a BglII site (5’-GAGAAATGCCTGGGCTCTGGGGGCCTGAGA-3’). The amplified fragment was cut with NheI/BglII and cloned into mammalian vector pCEP4, in-frame with CD5L at the 5′ end and with the Fe portion of human IgG1 at the 3′ end (pCEP4/CD5L-AITR-Fc). pCEP4/CD5L-AITR-Fc was used to transfect HEK293 cells by the standard calcium phosphate precipitation method (pCEP4/CD5L-AITR-Fc). Twenty-four hours after transfection, cells were lysed with 1 ml of lysis buffer (50 mM HEPES (pH 7.4), 250 mM NaCl, 1% Nonidet P-40, 10% glycerol, and protease inhibitors). For immunoprecipitation, lysates were incubated with anti-Flag M2 (Eastman Kodak Co.) or control murine IgG1 mAb at 4 °C for 1 h, followed by incubation with 20 μl of a 1:1 slurry of protein G-Sepharose (Pharmigen, San Diego, CA) for another hour. Precipitates were thoroughly washed with lysis buffer, then fractionated on a 10% SDS-polyacrylamide gel before transfer to polyvinylidene difluoride membrane (Millipore). Western blot analysis was performed with anti-HA mAb coupled with horseradish peroxidase (Boehringer Mannheim) and visualized using the enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech).
transfected into HEK293 EBNA cells. AITR-Fc fusion protein was purified from pCEP4/CD5L-AITR-Fc-transfected HEK293 EBNA cell supernatants using the protein G column. To generate a Flag-tagged soluble form of TL6 protein (amino acids 39–169), the Flag-tagged TL6 expression vector (pCEP4/CD5L-TL6-Flag) was constructed by PCR amplification of TL6 coding sequences using sense (5'-CTAGCTAGGCACGGCCGCGCGCAGAAGAGACT GCTAGGCCGCGCGCGCGCGCGGCTAGCTACAAAGG CGAGCTGACTAAGGAC GCTGCC-3') and antisense (5'-CCGCTCGAGGCTTTACAGGAAGAGAGTAAAGAAGG GCTCC-3') primers, digesting the product with NheI/XhoI, and cloning into pCEP4, in-frame with the CD5L sequence. The construct was expressed in HEK293 EBNA cells. Transfected cell supernatants containing secreted TL6-Flag were harvested and used for binding assays.

For some experiments, TL6-Flag protein was purified from harvested supernatants, using anti-Flag gel (Sigma) according to the manufacturer's instructions.

**Binding Assay**—Protein binding assays were done essentially as described (12). For cell-binding assays, HEK293 EBNA cells were transfected using pcDNA3.1/CD5L-AITR or pcDNA3.1, as described above. Forty-eight hours after transfection, cells were harvested and incubated consecutively with TL6-Flag-containing supernatant, anti-Flag antibody, and FITC-conjugated anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL). Flow cytometry analysis was performed using the Becton Dickinson FACSscan (San Jose, CA). Jurkat T cells were stably transfected by electroporation using linearized pcDNA3.1/CD5L-AITR and selected in the presence of Zeocin (Invitrogen). A binding assay for this cell line was performed as described above. To test the ability of AITR-Fc fusion protein to bind membrane-bound TL6, pCEP4/TL6 was stably transfected into HEK293 EBNA cells. After selection in the presence of hygromycin, TL6-expressing cells were harvested and incubated with AITR-Fc protein, followed by FITC-conjugated anti-human IgG1 antibody (Southern Biotechnology). The Becton Dickinson FACSscan was used for flow cytometry analysis.

**RESULTS AND DISCUSSION**

AITR was identified by searching an EST data base. A full-length cDNA of a clone from a human activated T-cell cDNA library, which is tentatively named AITR (for activation-inducible TNFR family member), encodes a 234-amino acid type I transmembrane protein with a calculated molecular mass of 25 kDa (Fig. 1A). The receptor has a signal peptide (the first 25 amino acids) and a single transmembrane region (amino acids 140–158). When compared with the extracellular domain of other TNFR family members, AITR displays three cysteine-rich pseudorepeats corresponding to the second, third, and fourth TNFR motif, respectively. The first cysteine pseudorepeat contains eight cysteine residues and lacks C4. Therefore, it is unlikely that the canonical pattern of C1-C2, C3-C5, and C4-C6 disulfide bridges exist in this motif. The second pseudorepeat shows some features of the third TNFR motif, but it is atypical in that C5 is not present even though it contains 7 cysteine residues. The third pseudorepeat shows extensive homologies with the fourth pseudorepeat of 4-1BB. The cytoplasmic domain contains acidic amino acids that are highly conserved in the cytoplasmic domains of 4-1BB, CD27, and GITR. Overall, AITR exhibits a high homology (55% identity) to murine GITR (Fig. 1B), but there is a mismatch in the first cysteine-rich pseudorepeat between GITR and AITR, because the first pseudorepeat of GITR corresponds to the first TNFR cysteine-rich motif (24).

We investigated expression of AITR mRNA in multiple human tissues by Northern blot hybridization (Fig. 1C). A 1.25-kilobase mRNA was detected in lymph node, peripheral blood leukocytes, and, weakly, in spleen. We also tested a variety of tumor cell lines for expression of AITR mRNA (Fig. 1C). A 1.25-kilobase message was detected only in the colorectal adenocarcinoma cell line, SW480, among the cell lines tested. The expression of virtually all members of the TNFR superfamily is expressed in lymphocyte activating ligand (1). Consistent with this idea, AITR expression was up-regulated in PBMC after stimulation. No AITR message was detectable in unstimulated PBMC when we used a sensitive RT-PCR method (Fig. 1C). AITR expression was clearly induced within 24 h by typical PBMC stimulation such as treatment with PMA plus ionomycin or soluble anti-CD3 plus anti-CD28 mAbs. FACS analysis for AITR expression, however, showed that a small population of activated PBMC expressed AITR on the cell surface at 48 h after stimulation, suggesting that a prolonged period of stimulation is required for maximum expression of
Expression of AITR was not induced by treatment with dexamethasone. This property was different from that of GITR (24).

Recently it has been shown that 4-1BB molecules associate with TRAF1, TRAF2, and TRAF3 (27–29). Because the cytoplasmic domain of AITR is similar to that of 4-1BB, we tested its ability to co-precipitate five of the six known TRAFs that were overexpressed in HEK293 EBNA cells. We observed an interaction of AITR with TRAF1, TRAF2, and TRAF3 but not with TRAF5 and TRAF6 (Fig. 2A). The association of AITR with TRAF2 suggested that, like other members of the TNFR superfamily (27, 28, 30, 31, 39–41), AITR might mediate NF-κB activation through TRAF2. To test this possibility, we used an NF-κB reporter system in HEK293 EBNA cells (31). Co-transfection with the AITR expression vector typically induced greater than 3-fold higher luciferase activity when compared with the vector transfection control (Fig. 2, B and C). When co-expressed with TRAF2, AITR induced greater luciferase activity than did TRAF2 alone (Fig. 2, B and C). More importantly, overexpression of dominant-negative TRAF2, which lacked the RING and zinc finger motifs (31), abrogated the luciferase activity induced by AITR (Fig. 2B). This indicates

FIG. 3. TL6 is the ligand for AITR. **A**, the extracellular domain of AITR selectively bound TL6. AITR-Fc protein was incubated with supernatants containing TL3-, LIGHT-, TL6-, TL7-, or 4-1BBL-Flag protein (DNA sequences of TL3 and TL7 not published). AITR-Fc-ligand complexes were precipitated with protein G-Sepharose and resolved on a 10% SDS-polyacrylamide gel. Bound ligand was identified by immunoblot. **B**, FACS analysis of AITR-TL6 interactions. Upper panel, HEK293 EBNA cells were transfected with pcDNA3/CD5L-AITR or an empty vector using the calcium phosphate method. Forty-eight hours after transfection, cells were harvested with 10 mM EDTA in phosphate-buffered saline, incubated with supernatant containing TL6-Flag, and stained with anti-Flag antibody, followed by FITC-conjugated secondary antibody. Middle panel, AITR-transfected and parental Jurkat cells were similarly stained as described above. AITR mRNA was detected in transfectant (AITR) but not in parental (P) cells by RT-PCR (inset). Lower panel, HEK293 EBNA cells were transfected with pCEP4/T6 or an empty vector using the calcium phosphate method. After selection in the presence of hygromycin, cells were harvested with 10 mM EDTA in phosphate-buffered saline, incubated with AITR-Fc fusion protein, and stained with FITC-conjugated anti-human IgG1 antibody. Expression of TL6 mRNA was confirmed in transfectant cells (inset). **C**, AITR/TL6-mediated NF-κB activation. HEK293 EBNA cells, which were stably transfected with an empty vector (pCEP4), a full-length TL6 (pCEP4/T6), or a Flag-tagged AITR extracellular domain construct (pCEP4/CD5L-AITR-Flag) were transfected with the pELAM-Luc reporter plasmid, pRSV-β-gal, and indicated combinations of expression vectors for AITR or an empty vector. Luciferase activities were measured after 24 h and normalized on the basis of β-galactosidase activity levels. Values are presented as mean ± S.E. Each experiment was done in triplicate, and the data presented are representative of three independent experiments.

AITR. Expression of AITR was not induced by treatment with dexamethasone. This property was different from that of GITR (24).

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B. Kwon, unpublished data.
Northern blots of poly(A) RNA from human tissues and HUVEC cells. A, expression of TL6 in human tissues and HUVEC cells. Hybridization to a 32P-labeled cDNA sequence of TL6. The potential trans-

A

analysis of TL6.

that TRAF2 is an important mediator of NF-κB activation for AITR. A similar observation was made when we blocked the activity of NIK, which was thought to lie downstream of TRAF2 in the NF-κB signaling pathway, by overexpression of the dominant-negative NIK (36), which lacked the two lysine residues of the catalytic domain (Fig. 2B). Taken together, these data indicate that AITR mediates NF-κB activation through the TRAF2/NIK pathway. Since TRAF1 and TRAF3 were found to associate with AITR in HEK293 EBNA cells, we examined the effects of TRAF1 and TRAF3 on NF-κB activation induced by AITR. As shown in Fig. 2C, introduction of TRAF3 nearly abolished the luciferase activity induced by AITR overexpression. To a lesser extent, TRAF1 overexpression diminished AITR-induced NF-κB activation. These data suggest that TRAF1 and especially TRAF3 down-regulate AITR-induced NF-κB activation.

To identify AITRL, we screened a panel of Flag-tagged candidate TNF ligand proteins for binding to AITR-Fc fusion protein by immunoprecipitation. AITR-Fc selectively bound TL6-Flag among Flag-tagged TNF ligand proteins tested (Fig. 3A). In our experimental conditions, 4-1BB and TR2 (HVEM) bound their cognate ligands, 4-1BBL and LIGHT (38), respectively (data not shown). Furthermore, our data clearly showed that TL6-Flag protein bound AITR transiently expressed on the cell surface of HEK293 EBNA cells and AITR constitutively expressed on the cell surface of Jurkat cell (Fig. 3B, upper and middle panels, respectively). Since TL6 is a transmembrane protein (see below), we used flow cytometry to determine whether AITR-Fc fusion protein was able to bind HEK293 EBNA cells that were stably transfected with full-length TL6. We found that AITR-Fc protein was capable of binding TL6 expressed on HEK293 EBNA cells (Fig. 3B, lower panel). Next, we tested whether interactions between AITR and TL6 would result in NF-κB activation. In an NF-κB reporter assay, ligand-dependent NF-κB activation was demonstrated by co-transfecting transmembrane TL6 with AITR (data not shown) or transfecting TL6-expressing HEK293 EBNA cells (Fig. 3C). In addition, when AITR was transiently transfected into HEK293 EBNA cells, which constitutively secreted soluble TL6 protein, NF-κB activation markedly increased as compared with empty vector-transfected HEK293 EBNA cells (Fig. 3C). Similarly, higher NF-κB activation was induced by treating with soluble TL6 protein HEK293 EBNA cells that were transiently transfected with AITR (data not shown). This indicates that TL6 is able to trigger AITR-specific activation of NF-κB. It appears that higher induction of NF-κB by TL6 is correlated with a stronger association of AITR with TRAF2 in HEK293 EBNA cells, since stronger association of AITR with TRAF2 was observed in cells that were co-transfected with TL6 than in cells that were transfected with AITR alone.

TL6 was one of the TNF ligand proteins initially identified by an EST data base search. Hydrophilicity analysis of a full-length TL6 clone from a brain cDNA library predicts a single hydrophobic transmembrane domain and the absence of a signal sequence (Fig. 4A). TL6 contains two potential glycosylation sites in the C-terminal region. These features suggest that TL6 is a type II membrane protein with the C-terminal region extracellular. Northern blot analysis of human tissue RNAs revealed expression of a single 2.4-kilobase TL6 mRNA in pancreas (Fig. 4B). Various human cell lines and PBMC were also examined for TL6 expression. No message was detectable in either unstimulated or stimulated T-cell lines (CEM-6 and Jurkat), B-cell lines (Priess and Frey), promyelocytic cell line (HL-60), monocyte cell line (THP-1), and PBMC by RT-PCR (data not shown). In contrast, HUVEC cells constitutively expressed TL6, and its expression was up-regulated after stimulation with LPS (Fig. 4B). Therefore, it is speculated that AITR and its ligand are important for interactions between activated T lymphocytes and blood vessels.

AITR has 55% identity with murine GITR at the amino acid level. The high sequence conservation between human and mouse provides evidence that AITR is the human homologue of murine GITR. At this point, however, the possibility remains that these two receptors may serve distinct functions from one another, based on the following facts. 1) There is a mismatch in the first cysteine-rich pseudorepeat between GITR and AITR; 2) in contrast to GITR, AITR is not inducible by dexamethasone.

In summary, we have identified a novel protein of the TNFR superfamily, AITR, which activates NF-κB through a TRAF2-mediated mechanism. Expression of AITR is activation-inducible. The ligand for AITR is a member of the TNF ligand family and is constitutively expressed in an endothelial cell line. This indicates that AITR and its ligand may be involved in activated T cell trafficking.

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