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Regulation of the murine TR2/HVEM gene expression by IRF

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Abstract

TR2 (TNFR-related 2, HVEM, or TNFRSF-14), a member of the TNFR family, is involved in a number of immune responses. While TR2 is expressed on the surface of T cells during the resting state, little is known regarding how expression of the TR2 gene is regulated. To understand the mechanisms regulating the expression of TR2 in T cells, we analyzed the 5' flanking region of TR2. We identified an important region for the activity of the TR2 promoter using site directed mutagenesis. Using EMSA analysis, we found that IRF-2 was bound to the promoter region of the TR2 gene during the resting state of EL-4 T cells. Transfection of IRF-2 expression plasmid and of dominant negative IRF-2 mutant further confirmed our results. Together, these data demonstrate that IRF-2 is involved in the regulation of TR2 expression in EL-4 T cells.

Keywords: TR2/HVEM; T cell; Regulation; Promoter; IRF-2

Members of the tumor necrosis factor receptor (TNFR) family play important roles in immune regulation. The members of this family interact with a set of ligands that form the TNF family, and signals induced by these interactions are involved in a diverse array of processes including differentiation, proliferation, activation, or induction of apoptotic cell death [1].

To achieve optimal antigen receptor-mediated T cell activation, two different signals are necessary, namely, an initial signal delivered from TCR and the second from costimulatory molecules. The receptor-ligand pairs that transduce these secondary signals include B7/CD28, as well as TNFR family members CD40-CD40L, 4-1BB/4-1BBL, and LIGHT-TR2 [2]. TR2 (TNFR-related 2, also known as HVEM or TNFRSF-14) has been reported to be one of the costimulatory molecules, and its ligand is known as LIGHT (TL5 or TNFSF-14) [3]. LIGHT, a member of the TNF family, is capable of binding to herpes virus entry mediator (HVEM, TR2), lymphotoxin beta receptor (LTβR), and soluble decoy receptor 3 (DcR3) [4], all of which are members of the TNFR family. TR2/HVEM is expressed in various tissues and immune cells including T cells, B cells, and DC cells. TR2 signals promote cellular growth, proliferation, and the production of cytokines such as IL-2, IFN-γ, and TNF-α [5]. In addition, costimulation LIGHT for CTL activity of CD8+ T cells is involved in immune responses in tumors, GVHD [6], and graft rejection [7] via TR2 (HVEM).

Abbreviations: TNFR, tumor necrosis factor receptor; TR2, TNFR-related 2; HVEM, herpesvirus entry mediator; IRF, interferon regulatory factor; LIGHT, lymphotoxin homolog, which exhibits *i*nducible expression and competes with HSV *g*lycoprotein D for *H*veA and is expressed on *T*-lymphocytes.

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Although much has been discovered regarding the biological role of TR2, little is known about the regulation of TR2 gene. While some studies have shown that TR2/HVEM is constitutively expressed on the surface of resting T cells, a mechanism explaining how this occurs remains obscure [8]. To gain insight into these matters, we cloned a cDNA of the mTR2 gene and analyzed the 5′ flanking region. Our results indicated that IRF functions as a positive regulator of TR2.

Materials and methods

Isolation of phages containing the mTR2 gene. We cloned a complete mTR2 cDNA from the total spleen RNA of a mouse using a probe based on a sequence described previously [9]. The sequence of the obtained DNA was identical to that reported in NCBI (NM_178931) except for the 5′ untranslated region. We screened the mouse genomic library derived from strain 129/SvEv (Stratagene) using the cloned cDNA as a probe. A positive phage was hybridized with an mTR2 probe and the clone was sequenced after sub-cloned. Using BlastN software (NCBI) to look for related genes [10], we found that the murine genomic DNA sequence from clone RP24-89N4 on chromosome 4 (BX004788.7), published during the preparation of this manuscript, was identical to our sequence.

Mapping the transcription start site of the TR2 gene. The transcription start site of the murine TR2 gene was mapped with the 5′-RACE system (Invitrogen) according to the manufacturer's protocol. Briefly, total spleen RNA was reverse transcribed using the GSP1 primer (5′-ACT GTTCCACATGCTTGCA-3′) and PCR was performed with a poly-C tailed with a 5′-RACE abridged anchor primer and the GSP2 primer (5′-TGTGCTGACTGCCTAACAGGG-3′). Nested PCR was performed with a 5′-RACE abridged universal amplification primer and GSP3 primer (5′-GTGTCCATCCTTTTGCCAC-3′). The amplified products were cloned into the pGEM-T easy vector and sequenced.

Preparation of reporter gene construct and expression vectors. The genomic sequence (−2165 bp/−3, relative to ATG) upstream of the murine TR2 gene was isolated by PCR using the following primers: 5′-AAAGAGCTCCCACAGATATGGAAACA-3′ (sense, 1U) and 5′-CTCGAGTCTTGATCAAGAAACTTCAGG-3′ (antisense, 1D). The resulting PCR product was first cloned into the pGEM-T Easy vector and then into the reporter vector pGL2-Basic (Promega) to yield pTR2(−2165/−3)-luc. Additional constructs and sense primers used were pTR2 (−462/−3)-luc, 5′-GAGCTCGGGATAACCCTGACGTGGTGT-3′(2U) pTR2 (−276/−3)-luc, 5′-GAGCTCGGAGACAGACATACTTCCAGA-3′(3U) pTR2(−141/−3)-luc, 5′-GAGCTCGCTCCATCTGCCTCTAAC-3′(4U) pTR2(−104/−3)-luc, 5′-GAGCTCTATTTTTTCTTTCATTTTC-3′(5U) and pTR2(−79/−3)-luc, 5′-GAGCTCCTTGCCAAGGCTCATGA-3′(6U), respectively. The amplified cDNA fragments were cloned into pGL2 Basic vector. Whole nucleotide sequences from these constructs were confirmed by sequencing.

To investigate the binding of IRF-2 to the TR2 promoter, IRF-2 expression plasmids were prepared in pcDNA3. The IRF-2 (NM_008391) gene was cloned by PCR using total RNA from EL-4 cells and following primers: 5′-AAGCTTCCTTGCGGGATTGTATTGGTAG-3′ (sense) and 5′-GGGCCCGGCTGGAGTCCTGAGTTA-3′ (antisense). Dominant negative IRF-2 mutants were derived as follows: the IRF-2Δ123 insert, which represents 123 amino acids of the IRF-2 N-terminus, was generated by PCR using a plasmid expressing the full length IRF-2 gene and the primers 5′-AAGCTTCCTTGCGGGATTGTATTGGTAG-3′ (sense) and 5′-GGGCCCTTATTTTTCTGTCTTTGG-3′ (antisense) [11]. An expression plasmid for IRF-2Δ123 was constructed by cloning the insert into the ApaI/HindIII sites of the pC3.0 vector. The SacI, ApaI, HindIII, and XhoI sites of the above primers are underlined.

Cell culture and transfection experiments. EL-4 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum. For transfections, 10^7 EL-4 cells were electroporated at 950 microfarads and 300 V using a Bio-Rad Gene Pulser with 25 μg of the pGL2/basic vector or various TR2 promoter-driven luciferase constructs and 5 μg of the beta-galactosidase plasmid. Luciferase activity was determined using the Promega luciferase reporter assay and measured with a Dynex luminometer. Transfection efficiency was normalized to beta-galactosidase activity. The reported values represent the average of three independent transfections with standard deviation.

Electrophoretic mobility shift assay (EMSA). The nuclear extracts from aliquots of 10^7 EL-4 T cells were subjected to EMSA using a labeled probe with [γ-^{32}P]ATP generated with T4 nucleotide kinase. The reaction mixture was loaded directly onto a 6% non-denaturating polyacrylamide gel and electrophoresed at a constant voltage of 200 V at 4 °C. The double stranded oligonucleotides used were as follows: for the wild type IRF binding site in the TR2 promoter (P2), 5′-TTTTTTCTTTCATTTTCACT-3′, and its mutated homologue (P2M), 5′-TTTTTTCTAACAAATTCACT-3′; for the standard IRF binding probe, 5′-GGAAGCGAAAATGAAATTGACT-3′ (Santa Cruz). For supershift assays, 1 μg of monoclonal antibodies against IRF-1 (sc-640×, Santa Cruz), IRF-2 (sc-498×), IRF-3 (sc-9082×), IRF-4 (sc-6059×), or IRF-8 (sc-6058×) were added to the binding reaction mixture at room temperature prior to electrophoresis. The reaction mixture was loaded directly onto a 5% non-denaturating polyacrylamide gel and electrophoresed at room temperature at a constant voltage of 70 V.

ChIP assay. ChIP assays were performed as described previously [12]. Briefly, EL-4 cells were lysed and the nuclei were prepared. Chromatin was sheared incubated with the indicated antibodies: monoclonal antibody against IRF-1, -2, -3, -4, and -8, as in EMSA analysis. Immune complexes were collected with salmon sperm-saturated protein A and the DNA was extracted by phenol/chloroform, and was used for PCR. The primers used for mTR2 promoter were 5′-GGAGACAGACATACTTCCAGA-3′(−207 to −187), and 5′-TCTTGATCAAGAAACTTCAGG-3′ (−23 to −3).

Results

Identification and cloning of the murine TR2 gene 5′ flanking sequence

TR2/HVEM is expressed constitutively on the surface of T-cells in the resting state. To investigate the mechanism by which TR2/HVEM is expressed in resting T-cells, we cloned the cDNA of murine TR2 from the total spleen RNA of a C57/BL6 mouse based on a previously published sequence [9]. We identified the transcription initiation site of the mTR2 gene using 5′-RACE (see Materials and methods), and found that the 5′-untranslated region of the mTR2 was split by an intron. The two exons resulting from the split are underlined in Fig. 1A. The presence of mRNA was confirmed by RT-PCR using total RNA from various tissues (Fig. 1) and the following primers: 5′-AAAGAAGGCTGGCTCCTGTTC-3′, (sense, Ue, Fig. 1A) and 5′-CTGAAGGTGTTGTCTGTAGGG-3′ (antisense, De, Fig. 1), which span the first and second exons.

Functional characterization of the 5′-region flanking the murine TR2 gene

To investigate the regulation of mTR2, we cloned various segments of the 5′-region flanking the mTR2 gene and transiently transfected the resulting constructs into EL-4 cells. The largest reporter gene (−2165/−3 bp, 1U, Fig. 2A) induced substantial luciferase activity and deletion of the upstream DNA elements, though −276 bp (2U, and