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Letter to the Editor

Identification of three novel mRNA splice variants of GITR

Dear Editor,

GITR is a gene belonging to the nerve growth factor-tumor necrosis factor receptor (NGF/TNFR) family that is preferentially expressed in lymphoid tissues, is overexpressed following dexamethasone treatment or T cell receptor (TCR) stimulation and is able to modulate T cell apoptosis.[1] Like other members of the NGF/TNFR family, GITR is composed of an intracellular domain, a transmembrane domain and an extracellular domain that is crucial for the binding with the specific ligand.[2,3] In particular, the GITR extracellular domain, like other family members, comprises three pseudorepeats of approximately 40 amino acids containing 4–6 cysteine residues. Moreover, murine GITR gene comprises five exons. The first three exons code for the extracellular domain, exon 4 codes for a small part of the extracellular domain, the transmembrane domain and part of the cytoplasmic domain while exon 5 codes for the cytoplasmic domain (see Figure 1A).[4]

Several members of NGF/TNFR family are characterized by splicing variants.[5,6] We performed experiments to analyze whether different splicing products of GITR could be expressed in T cells. We first analyzed a number of clones isolated by a library screening of a hybridoma T cell line,[1] and found clones different from GITR mRNA. In particular, one of the isolated clones (GITR-B) showed 11 bases added between exon 4 and exon 5, belonging to the 3′ end of intron 4 (Figure 1A). Thus, in GITR-B exon 5 is 11 bp longer than the exon 5 found in GITR. Moreover, the 3′ intron/5′ exon boundary is flanked by the consensus splice site sequence CAG/N. Of note, the protein putatively coded by GITR-B is different in the cytoplasmic domain (Figure 1B), due to the reading frame shift. In particular, a region of this cytoplasmic domain (see Figure 1B) has a significant homology with a cytoplasmic region of CD4 and CD8 receptors known to bind $p56^{lck}$ kinase.[5]

By performing RT–PCR using a DNAse-treated RNA from normal thymocytes, another GITR splicing was observed (GITR-C, Figure 1A). In GITR-C, the intron between exon 4 and exon 5 is not spliced out. To ensure that the cloned PCR product derived from mRNA and not from DNA still present after DNAse treatment, we also performed RT–PCR experiments with a forward primer located in the exon 3 and demonstrated that the GITR-C is indeed a GITR splicing (not shown). In fact, intron 3 resulted spliced out while intron 4 was not. The protein putatively coded by GITR-C is different from GITR and GITR-B in the cytoplasmic domain, due to the addition of 67 bp (intron 4) and a reading frame shift (Figure 1B). This cytoplasmic domain does not show any significant homology with other known proteins.

Soluble forms have been described for almost all members of the NGF/TNFR family, as the result of proteolitic shedding or mRNA splicing.[6–9] To investigate whether any alternative splicing variant of GITR gene could produce a soluble form, some RT–PCRs were performed with primers located in the exons 3 and 5, using RNA derived from thymocytes as template. We cloned a new GITR splicing (GITR-D, Figure 1A), that lacks exon 4. The protein putatively coded by GITR-D (Figure 1C) is identical to GITR in the NH_2-terminal residues, comprising the signal peptide, the first and the second pseudorepeat and part of the third pseudorepeat motif truncated after the second cysteine, of the extracellular domain, and lacks the transmembrane and intracellular domains. Thus, GITR-D is a secreted protein which could bind the GITR ligand and function as a decoy receptor.

We also performed experiments to analyze the expression level of GITR splicings in normal cells. Results indicate that in thymocytes the amount of GITR-D mRNA is comparable to the amount of GITR mRNA, while the levels of GITR-C and GITR-B are lower (see Figure 1D,E). Therefore, we can hypothesize that GITR and GITR-D proteins are expressed at similar levels in resting T cells so that GITR-D could efficiently compete with GITR for the ligand binding. GITR-C and GITR-B, although expressed at lower level, might contribute to regulate T-cell activation and death by transduction pathways different from the ones activated by GITR. As an example, GITR may bind TRAF2 through the PxEE motif[10] and GITR-B may bind $p56^{lck}$ kinase through the CXC motif[5] (Nocentini *et al.* manuscript in preparation) thus cooperating in lymphocyte activation. We have previously demonstrated that the expression of GITR gene increases about fourfold in ConA activated thymocytes.[1] However, the amount of the PCR product deriving from GITR increases in the activated thymocytes, while the PCR product deriving from GITR-D decreases as a result of the increased competition by GITR in the PCR assay (Figure 1D, lane 2). This change of the GITR/GITR-D ratio might have a physiological role and suggests that in activated cells GITR-D may be less able to compete with GITR so that the GITR binding with its ligand might be favoured.

In conclusion, the four different splicing products of murine GITR gene originate a complex receptor system. The balance among the expression levels of the alternative splicings change with the T-cell activation stage and might result in the activation of different intracellular pathways, upon ligand binding, and modulate the triggering of GITR receptors through the modulation of the amount of free-ligand by means of the decoy target. Future studies will be devoted to further analyze the possible role of the alternative splicings of GITR in the regulation of T cell activation and death.

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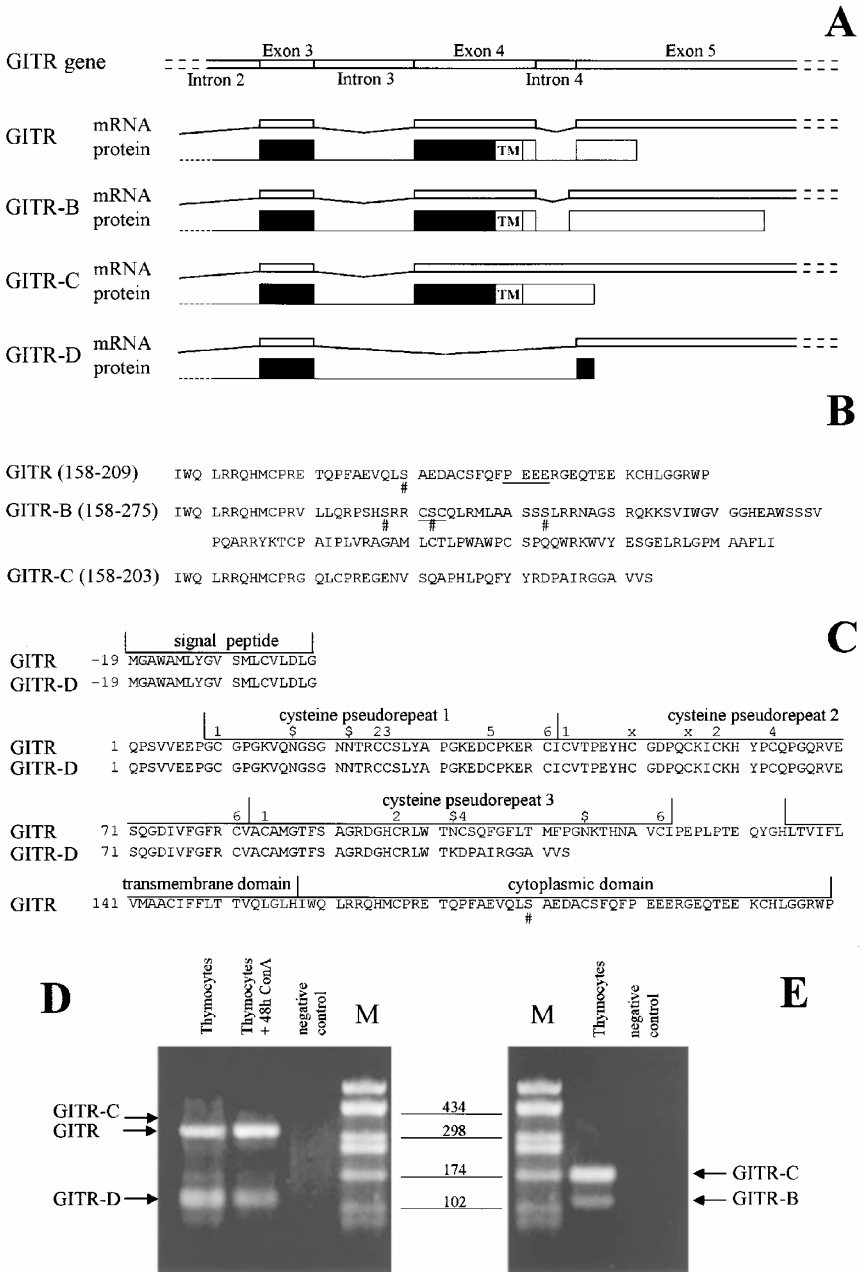

Figure 1 (**A**) Comparison of the exonic structure of GITR and its alternative splicings (exon 1 and 2 are not shown because identical). The putative proteins (-COOH terminal sequence is here reported) are characterized by an extracellular domain (black boxes), a transmembrane domain (empty boxes labeled with TM) and different cytoplasmic domains (gray boxes) with the exception of GITR-D that lacks the transmembrane domain. (**B**) Comparison among the cytoplasmic domains of GITR, GITR-B and GITR-C. The amino acidic regions that are highly homolog to other proteins, are shaded (GITR with 4-1BB, OX40 and CD27; GITR-B with CD4 and CD8) and the motifs potentially binding TRAF2 (GITR) and $p56^{lck}$ (GITR-B) are underlined. (**C**) Comparison between GITR and GITR-D protein (identical amino acids are shaded). Potential glycosilation ($) and phosphorylation (#) sites are shown. The cysteine pseudorepeats and the positions of the cysteine characterizing the repeats (from 1 to 6 for cysteines from the first to the sixth position, and x for the extra cysteines, as referred to the canonical repeat) are also reported. (**D**) PCR products obtained with a primer located on exon 3 and one located on exon 5. In lane 4 pUC18 *Hae*III digest was run for comparison. The length of the expected PCR products was 379 bp for GITR-C (which, however, is hardly visible due to the competition by GITR and GITR-D which are expressed at a higher level), 323 for GITR-B (which is not visible), 312 bp for GITR and 109 bp for GITR-D. cDNA were normalized by performing a PCR with primers specific for β-actin. (**E**) PCR products obtained with a primer located on exon 4 and one located on the boundary intron 4/exon 5. In lane 1 pUC18 *Hae*III digest was run for comparison. The length of the expected PCR products was 166 bp for GITR-C and 110 bp for GITR-B

Acknowledgments

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