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Identification of Three Alternatively Spliced Variants of Human CD28 mRNA

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CD28, expressed by T cells, plays a central role in providing costimulatory signals to T cells. The *cd28* gene is organized into 4 exons. An alternatively spliced CD28 mRNA lacking most of the exon 2 has been previously evidenced. We report here that non stimulated human T cells express three additional alternatively spliced variants of CD28 mRNA (CD28a-c) in. The CD28a variant, expressed at similar levels to that of the full length CD28 mRNA encoding for the membrane form, lacks exon 3. This deletion introduces (i) a frame shift resulting in the addition of two extra amino acids and a premature stop codon and, (ii) induces the loss of the transmembrane region, suggesting that it could encodes for a soluble monomeric molecule which conserves the binding sites of CD28. The CD28b and CD28c variants, expressed at a low level compared with CD28a, are generated by deletion of most of the 3′ end of exon 2 plus exon 3 and exon 2 plus exon 3, respectively. Activated T cells express only the membrane CD28 mRNA. These results suggest that resting human T cells may constitutively express both membrane and soluble CD28 which can differentially regulate the outcome of the T cell response.

An efficient antigen specific T cell activation requires two signals: (i) the first signal is delivered by the antigenic peptide complexed with MHC molecules and, (ii) the second signal is non specifc and delivered by the interaction between CD80 and CD86 expressed on the APC with CD28 constitutively expressed on T cells [1]. The costimulatory signal is generated by the interaction of CD80 and/or CD86, expressed by APCs, with CD28 which is constitutively expressed by T cells [2]. This signal is necessary for the proliferation, activation and induction of the effector functions of antigen-specific T cells [3-7]. Blocking the interaction between CD28 and its ligands using neutralizing anti-CD28 or anti-CD86 mAbs induces T cell anergy [8] and inhibits the T helper response [9]. The important role played by CD28 in the generation of an antigen-specific T cell response is illustrated in CD28-deficient mice. Although the development of a cytotoxic T cells response is normal, the initiation of a Th response and Ig class switching are reduced in CD28 KO mice [10]. Moreover, T cells from CD28-deficient mice cannot be activated via the B7 molecules [11]. A similar defect in the priming of a T cell response has been observed in soluble CTLA-4-Ig transgenic mice where the CD28-CD80/CD86 interaction is blocked [12].

The human *cd28* gene is a single copy gene organized into four exons: the first of encodes the 5′ untranslated region and leader sequence, the second encodes most of the extracellular domain while the third and fourth encode the end of the extracellular domain and transmembrane region and the intracellular cytoplasmic tail, respectively [13]. CD28 is a type I transmembrane glycoprotein protein belonging to the Ig superfamily [14] forming a disulfide-linked homodimer in with each subunit containing a binding site [15]. It presents a V-like domain in the extracellular region containing the conserved motif MYPPPY in the CDR-3-like region. This motif is involved in the binding of CD28 to its ligands [16, 17].

The aim of this work was to search for new forms of CD28 by RT-PCR using cDNA from non-activated human peripheral blood T lymphocytes. The results show that resting T cells constitutively express three alternatively spliced variants of CD28 mRNA, one of which encoding a soluble form.

MATERIALS AND METHODS

Isolation and activation of human T cells. T cells were isolated from heparinized venous blood from healthy subjects. Briefly, PBMC were isolated by centrifugation on Ficoll Hypaque (Amersham Phar-

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macia, Uppsala, Sweden) and T cells were purified by rosetting with sheep erythrocytes. The purity of T cells was >95%, as assessed by FACS analysis using a FITC-labelled anti-CD3 mAb (Immunotech, Marseille, France) (data not shown). T cells were cultured in complete medium consisting of RPMI 1640 medium supplemented with 5% FCS and antibiotics (all from Life technologies, Cergy Pontoise, France) and were either unstimulated or stimulated with 10 ng/ml anti-CD3 mAb (clone OKT3; ATCC, Manassas, VA) and 10 ng/ml coated anti-CD28 mAb (Immunotech) for 12 hours.

Cloning and sequencing of CD28 mRNA variants. Total RNA was extracted using Trizol reagent (Life technologies) and polyA+ RNA was purified using oligo-dT coated magnetic beads (Dynal, Oslo, Norway) accordingly to the manufacturers recommandations. The first strand cDNA was synthesized using 2 μg of poly-A+ RNA by reverse transcription using an oligo-dT primer (Pharmacia). PCR reactions were performed with cDNA corresponding to 10 ng of polyA+ RNA, an enzyme mix containing KlenTaq-1 polymerase and a proof reading polymerase (Advantage PCR enzyme systems; Clontech) and primers designed to amplify the entire coding sequence of CD28: 5′-ATGCTGAGGCTGCTCTTGGCTCTCAAC-3′ and 5′-TCAGGAGCGATAGGCTGCGAAGTCGCG-3′ (predicted size: 662 bp). PCR was performed as follows: 94°C for 5 min, 30 cycles 94°C for 1 min, 60°C for 1 min and 72°C for 2 min followed by a final extension at 72°C for 5 min. The amplified fragments were size-separated on a 1% agarose gel and visualized by ethidium bromide. After excision from the gel, each amplified cDNA fragment was subcloned into a TA cloning vector (pCRII; InVitrogen, Leek, The Netherlands). Sequencing was performed using the ABI-PRISM Dye Terminator Cycle Ready Reaction kit (Perkin Elmer, Foster City, CA). The expression of CD28 mRNA in activated *versus* non activated human T cells was evaluated by PCR using the above mentionned primers. RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA (5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′).

RESULTS

Identification of alternatively spliced variants of CD28 mRNA. The amplification by PCR of the CD28 coding sequence reveals the constitutive expression of four transcripts by non activated human T cells, with sizes of approximatively 660, 550, 240 and 180 bp (Fig. 1a). The subcloning and sequencing of these cDNA fragments show that they encode alternatively spliced isoforms of CD28. The largest form (650 bp) showed a complete homology with the membrane CD28 (data not shown) [14]. The 550 bp form (CD28a) presents a deletion from nt 409 (starting from the ATG start codon) to 534. The 240 bp form (CD28b) has a deletion from nt 118 to 534. The 180 bp form (CD28c) has a deletion from nt 53 to 534. According to the genomic organization of the human CD28 gene [13], these cDNA are generated by alternative splicing of the CD28 mRNA where exon 3 (Fig. 2a), the 225 last nucleotides of exon 2 plus exon 3 (Fig. 2b) and both exon 2 plus exon 3 (Fig. 2c) are spliced out, respectively. In each case, the deletion results in a frame shift with the consequence of an altered translation reading frame which results in 2 extra amino acids before a translational termination at position 418 in CD28a (Fig. 2a) at position 127 in CD28b (Fig. 2b) and at position 61 in CD28c (Fig. 2c).

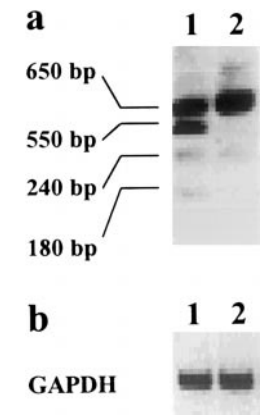

FIG. 1. Identification and regulation of alternatively spliced variants of CD28. (a) PCR amplification of CD28 cDNA. The coding sequence of human CD28 was amplified by PCR in non stimulated human T cells. The amplified fragments were size-separated on a 1% agarose gel and visualized by ethidium bromide (panel a, lane 1). (b) Regulation of CD28 mRNA expression in activated T cells. CD28 mRNA expression was evaluated in human T cells either unstimulated (panel a, lane 1) or stimulated for 12 h with anti-CD3 plus anti-CD28 mAb (panel a, lane 2). GAPDH cDNA was amplified as a control (panel b).

Regulation of CD28 mRNA expression in activated T cells. We report that resting T cells constitutively express four mRNA variants, the two larger being expressed at a higher level than the two smaller fragments. We have then evaluated the expression of the CD28 transcripts in activated T cells. A 12 h stimulation of T cells with anti-CD3 plus anti-CD28 mAb results in a preferential expression of the full length CD28 mRNA accompanied by a suppression of the spliced transcript expression (Fig. 1). Similar results were obtained after stimulation of PBMC with PHA or PMA plus ionomycin (data not shown).

DISCUSSION

We have identified three alternative spliced transcripts (CD28a-c) which are constitutively expressed by non stimulated human T cells in addition to the full length or membrane form of CD28.

The *cd28* gene is a single copy gene organized into four exons [13]. In the mRNA variants CD28a and CD28c, the acceptor and donor sites conforms with the consensus splice junction sequences [18]. However, in CD28b, while the 3′ acceptor site is identical to the consensus splice site CAG|G, the splice donor site located within exon 2 uses an unconventional splice sequence (CT|GCAAGT). The use of this splice site has been previously reported by Lee *et al* (1990). They reported the expression of four CD28 mRNA variants by non stimulated T cells of 3.7, 3.5, 1.5 and 1.3 kb. The difference between the 3.7/3.5 and 1.5/1.3 kb mRNA species is due to the use of an alternate, non-

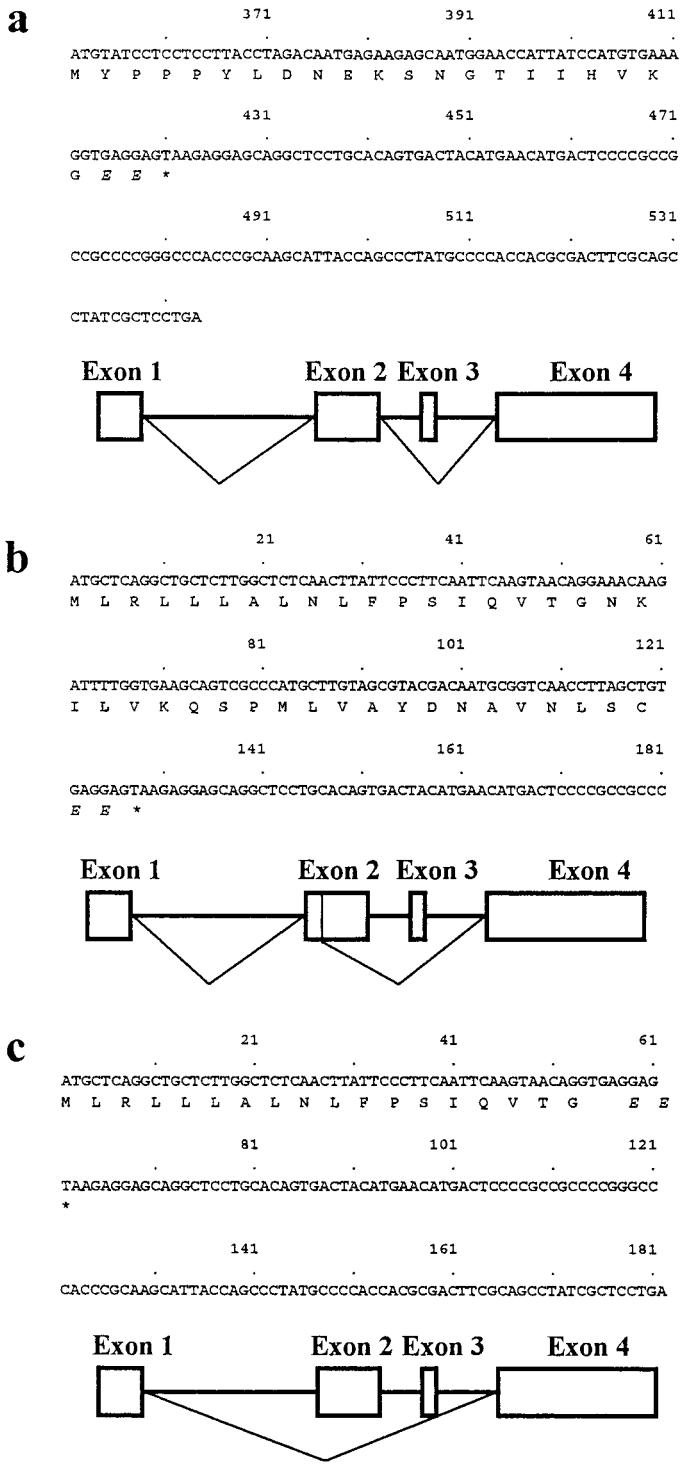

FIG. 2. Nucleotide sequences of the alternative splice variants CD28a-c. The 3′ end nucleotide sequence of CD28a (a) and the full length of CD28b (b) and CD28c (c) alternatively spliced variants of CD28 are shown. The stop codon is indicated by an asterisk. The predicted amino acid sequences are also presented and additional amino acids are denoted by italic letters. A schematic representation of the alternatively spliced trancripts is shown with the 4 exons depicted as white boxes.

consensus polyadenylation signal which results in the addition of 2.2 kb in the 3′ untranslated region. We did not detect the larger forms because we amplified only the coding sequence. The difference between the 3.7 *vs* 3.5 and the 1.5 *vs* 1.3 kb mRNA results from the deletion of a 252 bp fragment within exon 2, resulting in the loss of 84 amino acids in the extracellular domain. This deletion induces the loss of the V-like domain which contains the binding sites of CD28 to its ligands. It is interesting to underline that, even if the acceptor sites are different between the spliced variants reported by Lee *et al* and the one used to generate CD28b, the uncommon splice donor site they identified is the same used to generate the CD28b variant. Althought if we did not detect the alternatively splice variant described by Lee *et al*, collectively, these data suggest that resting human T cells could express at least five different CD28 mRNA variants.

The second exon in the CD28a variant remains intact, suggesting that the binding sites of CD28 located in the CDR1- and CDR3-like domains [16, 17] are conserved. Membrane CD28 is expressed as a disulfide linked homodimer, the dimerization being mediated by a cysteine residue located at position 141 [15]. The deletion of exon 3 induces the loss of this cysteine residue and the splicing induces a frame shift resulting in the loss of the intracellular domain. These results suggest that the truncated soluble CD28a molecule could be expressed as a soluble monomeric molecule by non activated T cells.

PCR analysis shows that the two bands corresponding to membrane CD28 and CD28a transcripts have similar intensities in non activated T cells. If CD28a is translated, both membrane and soluble CD28 molecules could be expressed at similar levels by resting T cells thereby suggesting that CD28a could play a role in the regulation of the immune response. Based on the current knowledge on the role of CD28 in the initiation of T cell responses (reviewed by Greenfiled *et al*, 1998), it is tempting to speculate that soluble CD28a could act as an inhibitory molecule that prevents the interaction between membrane CD28 and its counter-receptors. A previous study has reported that the interaction between a soluble CD80 molecule with its membrane ligand is different to the interaction between membrane CD28 and CD80/CD86 [5]. One can hypothesized that the avidity of soluble CD28 for membrane CD80/CD86 could be lower than the avidity of membrane CD28. Moreover, CD28 signaling is dependent on the avidity with its ligand [17]. Thus, additional experiments are required to compare the affinity and avidity of membrane *versus* soluble CD28 and the consequences on the amplitude of T cell activation. Interestingly, only the membrane CD28 transcript is expressed by T cells following stimulation with anti-CD3 plus anti-CD28 mAb. This result is in agreement with previous studies showing that activation increases the membrane expression of CD28 [3, 4, 19] and suggests

that the increase of CD28 expression is associated with an increase of CD28 transcription.

Our results show that resting T cells express several splice variants of CD28, including a preferential expression of membrane and soluble CD28 mRNA. Upon activation, T cells only express membrane membrane CD28 involved in the activation of T cells. In contrast, resting T cells may express both membrane and soluble CD28 which can share positive and negative regulatory roles in T cell activation, respectively.

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