LARD: A new lymphoid-specific death domain containing receptor regulated by alternative pre-mRNA splicing

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ABSTRACT Fas and TNF-R1 are cysteine-rich cell surface receptors related to the low-affinity nerve growth factor receptor family. Engagement of these receptors by their respective ligands, FasL and tumor necrosis factor, leads to apoptosis that is signaled through a conserved intracellular portion of the receptor termed the "death domain." We have cloned a new member of this family, lymphocyte-associated receptor of death (LARD), which leads to spontaneous apoptosis when expressed in 293T cells. The expression of LARD is more tightly regulated than that of either Fas or TNF-R1 as it is found predominantly on lymphocytes (T and B cells) but not on macrophages or a number of transformed lymphocyte cell lines. Alternative pre-mRNA splicing generates at least 11 distinct isoforms of LARD. The full-length isoform, LARD-1, extends to include the transmembrane and death domains, whereas the other isoforms encode potentially secreted molecules. Naive B and T cells express very little LARD-1 but express combinations of the other isoforms. Upon T cell activation, a programmed change in alternative splicing occurs so that the full-length, membrane-bound LARD-1 predominates. This may have implications for the control of lymphocyte proliferation following activation.

Programmed cell death or apoptosis is a general process that plays an important role in the development and function of a number of biological systems (1, 2). In the immune system, apoptosis is central to the establishment T lymphocyte repertoire, the control of lymphocyte proliferation, and cytotoxic T lymphocyte activity. Two cell surface receptors, Fas (APO-1, CD95) and TNF-R1 (tumor necrosis factor receptor 1; p55-R, CD120a) can trigger apoptosis. These two proteins contain cysteine-rich repeats that are also found in the low-affinity nerve growth factor (NGFR) family of proteins, which includes TNF-R2, CD27, CD30, CD40, OX40, and 4–1BB (3). A chicken receptor for avian leucosis virus, CAR1, is a recent addition to the family as it has two NGFR repeats and a death domain (4). A set of homotrimeric ligands for most of these receptors have been identified; these ligands define a further TNF-like protein family (3). The ligands for Fas and TNF-R1 are FasL and TNF, respectively. TRAIL, an orphan member of the TNF family, can induce apoptosis in certain target cells, although its receptor has not yet been identified (5).

Natural or targeted mutations of Fas or TNF-R1 demonstrate important roles in immune function. Fas may be involved in negative thymic selection, although mice with mutations in Fas (*lpr*), can still develop a normal T cell repertoire (6). Fas plays a part in cytotoxic T lymphocyte killing, which is underscored by the finding that mice deficient in perforin still show cytotoxic T lymphocyte activity, much of which is lost

when Fas is inhibited (7). Fas and FasL are up-regulated upon T or B cell activation, an event which targets may of these cells to apoptosis (8, 9). Fas mutations found in mice (*lpr*) or humans (Canale–Smith syndrome) or murine FasL mutations (*gld*) cause a syndrome of lymphadenopathy and autoimmunity resulting from unchecked lymphocyte expansions (3, 10).

Fas and TNF-R1 share homology in their cytoplasmic domains. This domain, which is sufficient to trigger apoptosis when expressed alone, has been termed the death domain. Yeast two-hybrid screens using the death domains of TNF-R1 and Fas have identified three interacting proteins, TRADD, FADD/MORT-1, and RIP, which also contain regions with homology to the death domain (11). The recent identification of FLICE/MACH-1, which interacts with FADD/MORT-1, provides a link to caspase activation (11).

These receptors account for some but not all the programmed cell death that occurs during normal immune maturation. In a search for further NGFR family members, we scanned the National Centre for Biological Information (NCBI) Expressed Sequence Tag (EST) database and identified LARD (lymphocyte associated receptor of death), which has a death domain and can initiate apoptosis. The potential importance of LARD in lymphocyte apoptosis is reflected by its restricted pattern of expression, predominantly on lymphocytes. In addition, we found that alternative pre-mRNA splicing generates at least 11 isoforms of LARD, providing a range of functional outcomes that may help shape the immune response. The down-regulation of LARD expression after lymphocyte transformation may allow escape from homeostatic processes designed to limit lymphocyte proliferation.

METHODS

Cloning of LARD cDNA. The NCBI EST database was screened for homology to the entire FAS molecule using the BLAST program. Several clones were identified; one of these clones (GenBank accession no. H41522) was requested and fully sequenced. This was found to be an 800-bp alternatively spliced clone that was used to screen a HeLa cDNA library. Two clones were isolated and sequenced and found to encode the LARD protein.

Analysis of LARD Expression. A full-length LARD cDNA was ^{32}P-labeled using a random primer kit (Primeit II, Stratagene). This probe was hybridized to multiple human-tissue Northern blots according to the manufacturer's instructions (MTN1-MTN2, Stratagene). RNase protection analysis was carried out according to standard protocols (12). A 266-bp complementary probe, covering the 3′ end of the LARD untranslated region, that will hybridize to all LARD isoforms

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RIP      I R E N L G K H W K N C A R K L . . . . . G F T Q S Q I D E I D H D Y E R D G L K E
TRADD    F A R S V G L K W R K V G R S L Q R G C R A L R D P A L D S L A Y E Y E R E G L Y E
FADD     I C D N V G K D W R R L A R Q L K . . . . . V S D T K I D S I E D R Y P R N . L T E
LARD     M D A V P A R R W K E F V R T L . . . . . G L R E A E I E A V E V E I G R . . F R D
TNF-R1   V E N V P P L R W K E F V R R L . . . . . G L S D H E I D R L E L Q N G R . C L R E
CAR1     V D Y V P F P E W K R F G R A L . . . . . D L Q E N D L Y L A E . Q H D R . V S C E
Fas      A G V M T L S Q V K G F V R K . . . . . N G V N E A K I D E I K N D N V Q D T . A E
Reaper   . . . . M A V A F Y I P D Q A T L L R E A E Q K E Q Q I L R L R . E S Q W R F L A T


RIP      K V Y Q M L Q K W L M R E G I K G A T V G K L A Q A L H Q C S R I D L L S S L
TRADD    Q A F Q L L R R F V Q A E G . R R A T L Q R L V E A L E E N E L T S L A E D L
FADD     R V R E S L R I W K N T E . K E N A T V A H L V G A L R S C Q M . N L V A D L
LARD     Q Q Y E M L K R W R Q Q Q P . . . A G L G A V Y A A L E R M G L D G C V E D L
TNF-R1   A Q Y S M L A T W R R R T P R R E A T L E L L G R V L R D M D L L G C L E D I
CAR1     P F Y Q M L N T W L N Q Q G S K . A S V N T L L E T L P R I G L S G V A D I I
Fas      Q K V Q L L R N W H Q L H G K K E A . Y D T L I K D L K K A N L C T L A E K I
Reaper   V V L E T L K Q Y T S C H P K T G R K S G K Y R K P . . . . . . . . . . . .
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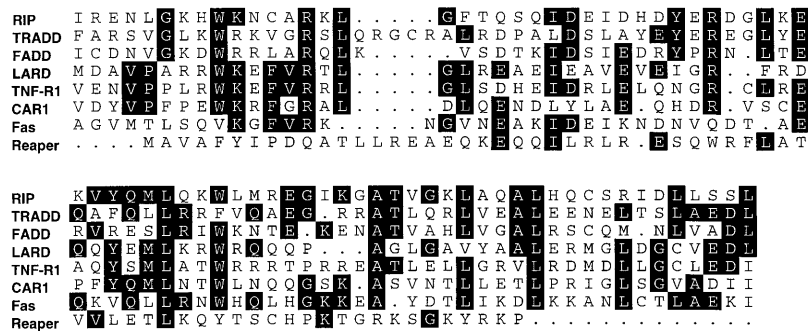
FIG. 1. Alignment of the death domain of LARD with the death domains of other proteins.

was generated from an SP6 promoter and hybridized with 10 μg of total cellular RNA.

Alternative Splicing. Peripheral blood lymphocytes (PBL) were prepared by Ficoll density gradient centrifugation of cells prepared from the buffy coat of 1 unit of human blood. CD4^{+}, CD8^{+}, and B cells were purified by absorption onto magnetic beads coated with antibodies to CD4, CD8, and CD19, respectively. Macrophages were prepared by adhesion to plastic plates for 30 min at 37°C. RNA was extracted using RNAzol B (Cinna/Biotecx Laboratories, Friendswood, TX). RNA (10 μg) was used in an oligo(dT)-primed cDNA synthesis using avian myeloblastosis virus reverse transcriptase. A 30-cycle PCR amplification was carried out with the primers F LARD Kpn, 5′-CGT AGG GTA CCC CAG GGC GGC ACT CGT AG, and R LARD Xba, 5′-GGC TCT AGA TCA CGG GCC GCG CTG CAG GC, which span exons 2–10. Products were cloned and sequenced, and a Southern blot was probed with ^{32}P-end-labeled primer F LARD Xba. A control PCR amplified a fragment from the glyceraldehyde-3-phosphate dehydrogenase cDNA.

Expression of LARD. An expression vector, pCD33L, was constructed in which the CD33 leader sequence was cloned into pCDNA3 (Invitrogen). A clone expressing full-length LARD was constructed by cloning a PCR product generated using the primers F LARD Kpn and R LARD Not, 5′-GCC AAA CTG CGG CCG CGA AGT TGA G, into pCD33L. To make the Fas/LARD chimera, the Fas extracellular and transmembrane domains were cloned into the *Kpn*I and *Eco*RI sites of CD33L using the primers F Fas Kpn, 5′-AAT GCG GTA CCT AGA TTA TCG TCC AAA AGT GTT AAT GCC C, and R FAS Eco, 5′-CAA GAA TTC TAC TTC CTT TCT CTT CAC CCA AAC AA. In a second step, the cytoplasmic domain of LARD was added by cloning a further PCR product generated with primers F LARD Eco, 5′-TGG GAA TTC ACC TAC ACA TAC CGC CAC TGC TGG, and R LARD Xba into the *Eco*RI and *Xba*I sites. Fas, TRAIL, and FasL expression vectors were constructed by cloning PCR products covering the entire coding regions of these genes into pCDNA3. Activity of FasL and TRAIL clones was checked by demonstrating that 293T transfectants could lead to apoptosis of Jurkat cells known to express both Fas and the receptor for TRAIL. CD8α, which was used in the cotransfection experiments, was cloned into the pREP-4 expression vector. 293T cells were transfected by calcium phosphate precipitation.

Analysis of Apoptosis. At 36 hr, the 293T transfected cells were selected with anti-CD8 coated magnetic beads (Dynal, Merseyside, U.K.). Cells were then removed from the beads using CD8 detachabeads according to the manufacturer's protocol (Dynal). A check for the efficiency of cotransfection was made for the Fas/LARD chimera by staining the CD8-purified 293T cells with the anti-Fas IgM mAb (Upstate Biotechnology, Lake Placid, NY), demonstrating that the majority of CD8-transfected cells also carried the FAS/LARD chimera.

CD8-purified cells were then cultured overnight, and cell death was assessed by trypan blue staining. Anti-Fas IgM mAb (Upstate Biotechnology) was used for the triggering of apoptosis.

RESULTS

Identification of LARD. In a search for new NGFR family members, we searched the NCBI EST database using the BLAST program to look for homology to Fas. A clone expressed in fetal brain showed homology to the cysteine-rich extracellular domain. This 800-bp clone has a termination codon halfway through the fourth NGFR repeat, which would be predicted to lead to a soluble LARD molecule. The termination codon is close to a sequence matching the consensus 5′ splice site, so we reasoned that it may represent an alternatively spliced variant of LARD. The partial-length cDNA was used to screen a HeLa cell cDNA library, and two further clones were obtained and sequenced, revealing a 417-aa ORF. The finding of these clones was fortuitous as RNase protection analysis shows very low expression of LARD in HeLa cells. As expected, LARD is highly homologous to both Fas and TNF-R1 (27% identity overall). The extracellular domain contains four complete NGFR repeats and two potential N-linked glycosylation sites. After a typical hydrophobic transmembrane domain, LARD has a 193-aa cytoplasmic domain. The death domain of LARD shows closer homology to TNF-R1 than to Fas (47% vs. 20% identity). Indeed LARD and TNF-R1 have complete conservation of the peptide RWKEFVR at the N terminus of the domain. The death domain of LARD is aligned with the death domains of other death domain-containing proteins (Fig. 1).

Tissue Distribution of LARD. Northern blot analysis using a full-length LARD cDNA probe reveals a message of 3.5–4 kb (Fig. 2). In contrast to Fas and TNF-R1, LARD expression is restricted predominantly to lymphoid tissues, spleen, thymus, and PBL. To quantify LARD expression on PBL subsets, we developed an RNase protection assay using a 3′ portion of the LARD cDNA as a probe. LARD is expressed in roughly equal amounts in B cells and CD4^{+} or CD8^{+} T cells (Fig. 3).
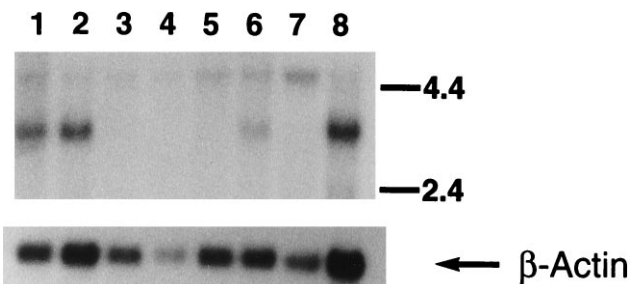

FIG. 2. Multiple-human-tissue Northern blot probed with LARD cDNA (*Upper*) and β-actin (*Lower*). Lanes: 1, spleen; 2, thymus; 3, prostate; 4, testis; 5, ovary; 6, small intestine; 7, colon; 8, PBL.
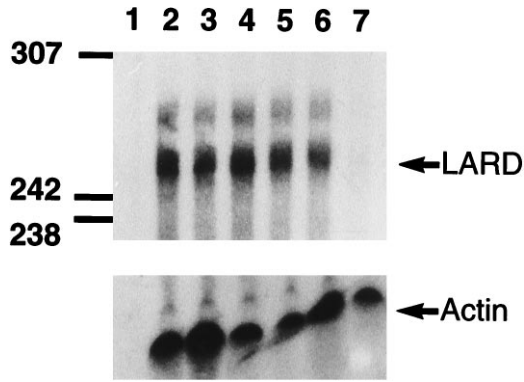
FIG. 3. RNase protection analysis on lymphocyte subsets. (*Upper*) LARD expression; (*Lower*) control protection using a β-actin probe. Lanes: 1, control (no RNA); 2, unstimulated PBL; 3, phytohemagglutinin (PHA)-blasted PBL; 4, CD4+ cells; 5, CD8+ cells; 6, B cells; 7, macrophages.

After PHA treatment of PBL, LARD mRNA expression is not increased. Interestingly, LARD is not expressed on macrophages, laboratory Epstein–Barr virus-transformed B cells, or the lymphocyte tumor cell lines (Jurkat, H9, HUT-78, C8166, Daudi, Raji, Namalwa, CIR, and HL-60), and very low level expression was seen on the T cell lines CEM and MOLT-4 and the colon cancer cell line HT-29.

Proapoptotic Activity of LARD. Overexpression of Fas or TNF-R1 can lead to apoptosis in the absence of their ligands, probably through homomultimerization of the death domains (13). LARD was cotransfected with CD8α into 293T cells, and after 48 hr, the transfected cells showed the typical morphological features of apoptosis, and cell death was assessed by trypan blue staining. Expression of LARD resulted in an 18% increase in cell death above background (Fig. 4*A*). To test the effects of receptor crosslinking and also to check for function of the death domain, a fusion between the extracellular domain of Fas and the intracellular domain of LARD was made. Receptor crosslinking with an anti-Fas mAb leads to an increase in cell death from 20% to 35% for the Fas/LARD chimera compared with 50% for cells transfected with full-length Fas and exposed to anti-Fas antibody (Fig. 4*B*). The increased apoptosis seen with the full-length Fas construct probably results from 3-fold higher expression on transfected cells when compared with the Fas/LARD chimera (Fig. 4*C*).

We have not yet been able to establish a stable cell line expressing LARD because of its propensity to cause spontaneous cell death. Neither the addition of TNF-α to LARD transfectants nor coculture with cells transfected with FasL or TRAIL leads to any increment in cell death (data not shown). This suggests that LARD is not the receptor for TNF-α, FasL, or TRAIL and has a novel, as-yet-unidentified ligand.

Alternative Pre-mRNA Splicing of LARD. The first LARD clone we sequenced was an alternatively spliced variant containing a retained intron (LARD-11), which leads to the insertion of a premature termination codon. For this reason, we initiated a more systematic search for alternative splicing using reverse transcriptase–PCR to amplify LARD cDNA from resting lymphocyte subsets and PHA-blasted lymphocytes.

LARD is extensively alternatively spliced, producing at least 12 distinct variants (Fig. 5). Alignment of these clones with the cDNA encoding the full-length LARD protein, LARD-1, shows that all but four are generated by the skipping of one or more portions of LARD-1. Comparison of these clones with the genomic structure of Fas (14) suggests the LARD variants are generated by the skipping of one or more complete exons. This comparison allows a tentative scheme for the genomic structure of LARD to be proposed; this structure probably
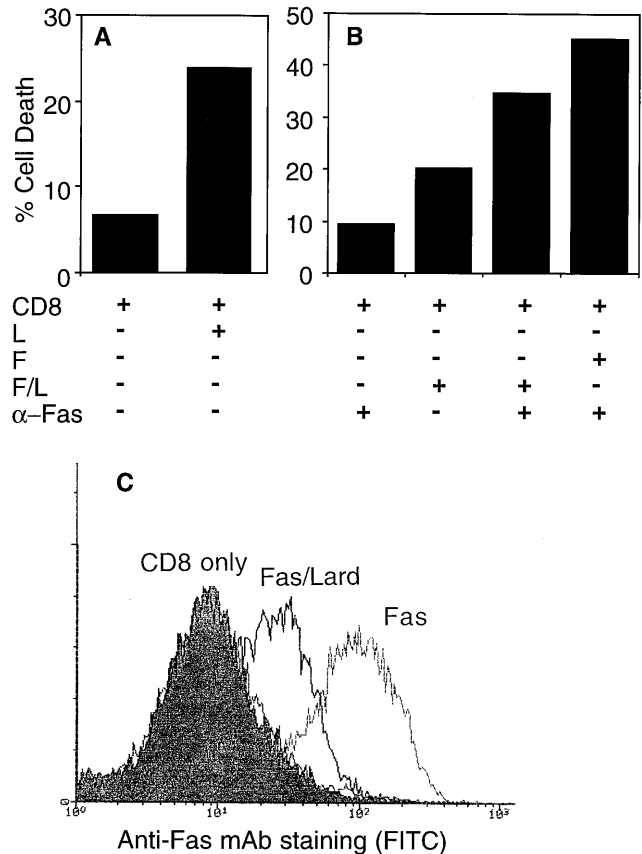

FIG. 4. Cell death was assessed by trypan blue staining and expressed as a percentage of the total. (*A*) Cells transfected with CD8 alone or cotransfected with CD8 and LARD. (*B*) effects of crosslinking with an anti-Fas mAb. F, Fas; L, LARD; F/L, Fas/LARD chimera. (*C*) Expression of Fas on transfected cells analyzed by a fluorescence-activated cell sorter. These data are representative of three separate experiments.

contains 10 exons, 6 for the leader and extracellular domain, 1 for the transmembrane domain, and 3 for the cytoplasmic domain (Fig. 5*A*).

Clone LARD-2 and LARD-10 have 101- and 200-bp insertions leading to premature termination proximal to the sequence encoding the transmembrane domain. Both inserted sequences have matches to consensus splice sites at their 5′ and 3′ ends and, therefore, likely represent short retained introns. Finally, clone LARD-1b contains an extra three nucleotides, CAG, inserted at position 709, which probably reflects splicing to an alternative 3′ splice site three nucleotides proximal to the one used in LARD-1. The rest of the variants are generated by skipping one or more of exons 3–8. Most of the alternatively spliced clones have stop codons introduced before the transmembrane domain. Only three variants, LARD-7, -8, and -9, extend into the cytoplasmic domain; LARD-7 and -9 skip the transmembrane domain, whereas LARD-8, a low abundance clone, only lacks a single NGFR repeat. In summary, apart from LARD-1b and LARD-8 these variant cDNAs encode isoforms with either 2.5 or 3.5 NGFR repeats which are preceded by the normal leader sequence but have no transmembrane domain and would, therefore, be expected to be secreted in a soluble and probably functional form.

Differential Expression of LARD Isoforms. Because there is no change in overall LARD expression in different lymphocyte subsets (see above), we looked to see if the splicing pattern varies during differentiation. After lymphocyte activation, there is a complete switch in splicing that will expose PHA-blasted cells to the risk of apoptosis triggered through LARD
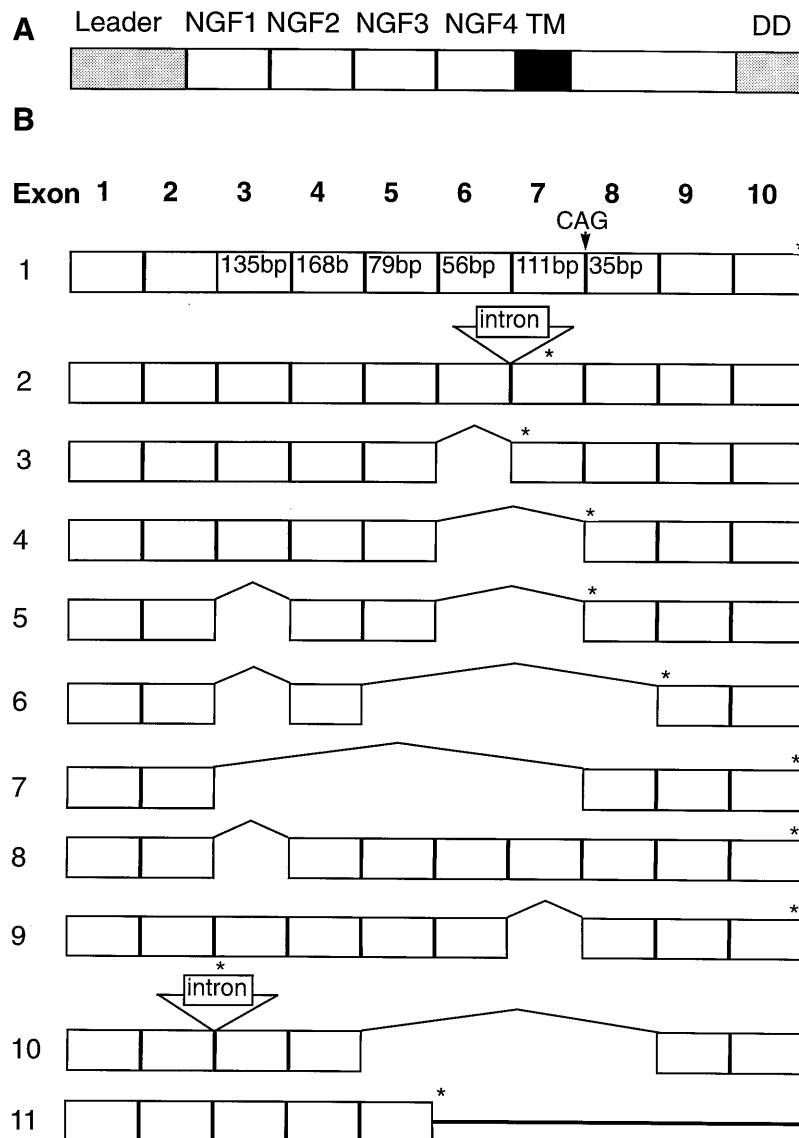
A Leader NGF1 NGF2 NGF3 NGF4 TM   DD

B

Exon 1   2   3   4   5   6   7   8   9   10

CAG

1 | | | 135bp | 168b | 79bp | 56bp | 111bp | 35bp | | |

intron

2

3

4

5

6

7

8

9

intron

10

11

FIG. 5. (*A*) Schematic representation of the LARD protein drawn above the proposed genomic structure showing the leader peptide, four NGFR repeats, the transmembrane domain, and the death domain. (*B*) A proposed scheme for the genomic structure of LARD is represented above the prototype LARD-1 cDNA. Alternatively spliced clones LARD-2 through -11 are represented below LARD-1. Positions of the termination codons are represented by an asterisk above the clones. The CAG insertion in clone LARD-1b is indicated above LARD-1.

(Fig. 6). Unstimulated B cells or CD4$^+$-CD8$^+$ T cells express very little LARD-1; instead, they express the truncated isoforms. The splicing pattern reverses after PHA blasting when isoforms encoding the truncated molecules are much reduced and LARD-1 predominates.

DISCUSSION

In this paper, we describe the cloning of LARD, the third death domain-containing member of the NGFR family. LARD shows close homology to TNF-R1 in the death domain, and there is no homology to the C-terminal 15 aa of Fas that are thought to play a protective role, through interaction with proteins such as FAP-1 (15). The close homology of LARD to TNF-R1 may indicate that it will signal through pathways more closely allied to TNF-R1 than Fas. Three papers have appeared very recently describing the cloning of the same molecule DR3, WSL-1, or APO-3 (16–18). In these reports, the intracellular interactions of the new death domain have been studied and found to more closely resemble TNF-R1 than Fas. We have characterized LARD expression on lymphocyte

subsets and cell lines and demonstrate that alternative splicing plays a key role in the expression of the full-length receptor after lymphocyte activation.

We show that crosslinking of Fas/LARD chimeras leads to an increase in death of transfected cells. This supports the concept that receptor crosslinking, normally achieved by interaction with a trimeric receptor, leads to multimerization of the death domains, and hence the formation of a more efficient platform for further signaling steps in the death pathway. Cells transfected with LARD do not show any increment in killing when exposed to the three death receptor ligands, TNF, FasL, and the orphan ligand TRAIL. This implies that LARD will have a unique ligand, the cloning and characterization of which will provide further insights into the function of LARD.

It is estimated that at least 10% of genes can be alternatively spliced, but in many cases the function of proteins produced remains obscure. The switch in LARD splicing that occurs upon T cell activation is a dramatic example of a case where alternative splicing may have important functional effects. The alternatively spliced clones encode molecules with either 2.5 or 3.5 NGFR repeats that are likely to be correctly folded (CAR1
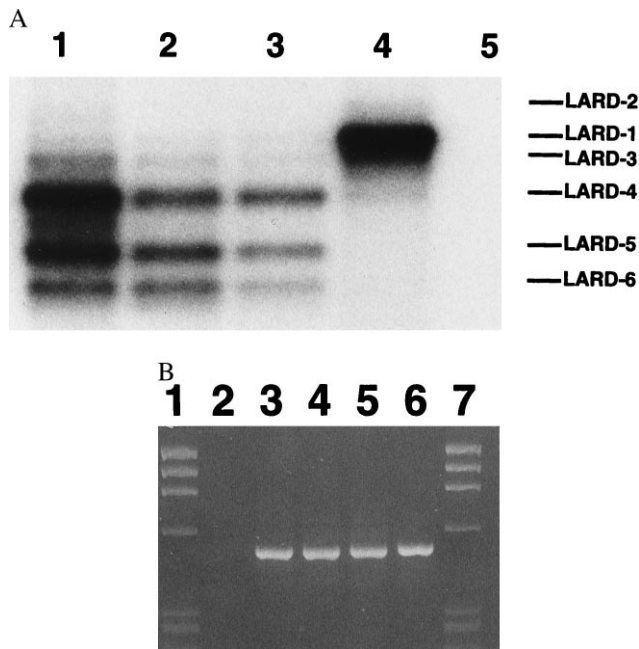
FIG. 6. (*A*) Southern blots of reverse transcriptase–PCR of LARD cDNA with primers F LARD Kpn and R LARD Xba probed with ^{32}P-labeled primer F LARD Xba. Lanes: 1, CD4$^+$ cells; 2, CD8$^+$ cells; 3, B cells; 4, PHA-blasted PBL; 5, negative control. (*B*) Ethidium-stained control PCR with glyceraldehyde-3-phosphate dehydrogenase primers. Lanes: 1 and 7, ϕX *Hae*III markers; 2, negative control; 3, CD4$^+$ cells; 4, CD8$^+$ cells; 5, B cells; 6, PHA-blasted peripheral blood mononuclear cells.

has 2 and Fas has 3 NGFR repeats) and secreted in a functional form. The likely functional nature of these isoforms is implied by ability of soluble Fas and TNF-R1, produced by alternative splicing or proteolytic cleavage, to bind to their ligands and block receptor signaling (19, 20). Hence the pattern of alternative splicing adopted by cells, by producing membrane anchored or soluble molecules, is likely to set the balance between susceptibility and protection from LARD-mediated apoptosis.

T cell activation leads to changes in the splicing of several cell surface proteins, CD44 and CD45 being notable examples (21, 22). A number of protein regulators of alternative splicing have been reported, including the eight members of the SR family of RNA binding proteins and hnRNP proteins A1, A1^b, and A2/B1 (22, 23). We have shown previously that the levels of some of the SR proteins change upon T cell activation, whereas hnRNP A1 levels remain unchanged (22). These protein factors would, therefore, be candidates for the control of the switch in LARD splicing seen in activated cells.

The restricted expression of LARD suggests that like Fas it may play a role in immune development and function. Our demonstration that LARD is up-regulated by alternative splicing in response to lymphocyte activation means that it is ideally placed to play a role in controlling lymphocyte proliferation. LARD expression is down-regulated on transformed lymphocyte cell lines, and it is interesting to speculate that the loss of LARD may be a key event in the development of lymphoid malignancies, allowing the escape of cells from homeostatic

processes in place to check lymphocyte proliferation in the absence of antigen. Finally, although Fas is involved in thymic selection, *lpr* mice do not show any abnormalities in T cell receptor repertoire, implying that there are other factors, maybe LARD, responsible for negative selection. Many of these questions may be resolved by the construction of LARD-deficient mice.

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