



Structure of the murine TRAF1 gene

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Abstract

We have cloned, characterized and sequenced the murine TNF Receptor Associated Factor 1 (TRAF1) gene. Restriction mapping and Southern blotting analysis revealed that the TRAF1 gene comprises 10 exons and 9 intervening introns and spreads over 18 kb of genomic DNA. 5'-RACE analysis of the TRAF1 transcript using mRNA from activated spleen B cells revealed several transcription start sites between positions -42 to +4 relative to the 5' end of the murine TRAF1 cDNA sequence. We also isolated and sequenced the 5'-upstream promoter region, which lacks TATA-like and CAAT-like sites but contains GC-rich sequences. Taken together, these results suggest that the TRAF1 gene promoter is a member of the class of Sp-1-dependent promoters. Near the transcription initiation start site we identified three identical decanucleotide repeats (CCAGCCAGC) which may play a role in the transcriptional regulation of TRAF1 expression. In addition we show that TRAF1 mRNA is not expressed in non-stimulated lymphocytes but can be induced upon activation with different stimuli, including anti-CD3, anti-IgM, anti-CD40 antibodies, LPS, or a combination of phorbol-12-myristate-13-acetate and ionomycin. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: TRAF1; Gene structure; Promoter sequences

1. Introduction

Members of the tumor necrosis factor alpha (TNF) superfamily are pro-inflammatory cytokines involved in a variety of physiological and pathological conditions. TNF superfamily members include TNF, lymphotoxins α and β , CD30L, CD40L, CD70, CD95/Fas, 4-1BBL, and others. They interact with the members of TNF receptor (TNFR) superfamily and play important roles in a wide array of biological processes including the acute phase response, cell growth and apoptosis, inflammation and lymphocyte activation (Baker and Reddy, 1996). The TNFR superfamily includes the following members: TNF-RI, TNF-RII, LT-R, CD27, CD30, CD40, CD95, OX-40, 4-1BB, and

others. All of these receptors are devoid of intrinsic catalytic activity. Some of them, like TNFR-I and CD95/Fas contain a death domain and bind to the recently identified signal transduction proteins like TRADD, FADD/MORT-1 and RIP. Other members of the TNFR superfamily, which lack a death domain and include TNFR-II, CD40 and CD30, associate with members of the TRAF (TNF Receptor Associated Factor) family of intracellular signal transduction molecules (Arch et al., 1998).

TRAF molecules were first described due to their ability to bind to TNF-RII (Rothe et al., 1994). Six TRAF molecules have been identified to date (Cao et al., 1996; Cheng et al., 1995; Hu et al., 1994; Ishida et al., 1996a, b; Mosialos et al., 1995; Nakano et al., 1996; Regnier et al., 1995; Sato et al., 1995). Individual TNFR family members may associate with one or several TRAFs, which in turn could result in a wide spectrum of divergent physiological functions. In general, TRAFs are characterized by the presence of an N-terminal RING finger, several zinc fingers and a

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C-terminal TRAF domain. The effector function of TRAF2 requires its finger domains whereas the TRAF domain is important for hetero- and homotypic interactions with receptors, other TRAF proteins, or other downstream signal transducers. It was shown *in vitro* that TRAF2 is required for JNK/SAPK and NF- κ B activation induced by two TNFRs, CD40, CD27, TRANCE-R, 4-1BB and CD30 (Akiba et al., 1998; Arch and Thompson, 1998; Gedrich et al., 1996; Horie et al., 1998; Jang et al., 1998; Lee et al., 1997; Natoli et al., 1997; Pullen et al., 1998; Rothe et al., 1995; Tsitsikov et al., 1997; Wong et al., 1998; Yamamoto et al., 1998). Gene targeting experiments demonstrated the crucial importance of the TRAF2 and TRAF3 proteins in cell signaling. Although TRAF2 null mice appear normal at birth, then become progressively runted and die prematurely. Examination of the TRAF2-deficient cells revealed a severe reduction in TNF-mediated JNK/SAPK activation but a surprisingly mild effect on NF- κ B activation (Yeh et al., 1997). Mice lacking TRAF3 exhibited a profound failure in homeostasis of the hematopoietic system and other organ systems, and die by 10 days of age (Xu et al., 1996).

TRAF1 is a unique member of the TRAF family. It does not have a RING finger domain and contains a single zinc finger, N-TRAF and C-TRAF domains. Originally, the murine TRAF1 was cloned by biochemical characterization of signal transducers associated with the cytoplasmic domain TNF-RII. It was purified from the lysate of the murine interleukin-2-dependent cytotoxic T cell line by immunoprecipitation with human TNF-RII (Rothe et al., 1994). Human TRAF1 (EB16) was cloned by association with Epstein–Barr virus latent infection membrane protein 1 (LMP1) from EBV-transformed lymphoblastoid cell line by the yeast two-hybrid screen (Mosialos et al., 1995). We and others have also cloned TRAF1 in addition to TRAF2 by yeast two-hybrid screening with the cytoplasmic domain of CD30 (Aizawa et al., 1997; Ansieau et al., 1996; Boucher et al., 1997; Duckett et al., 1997; Gedrich et al., 1996; Lee et al., 1996a; Tsitsikov et al., 1997). Ultimately, it was shown that TRAF1 can be recruited to a variety of distinct members of the TNFR superfamily, including TNF-RII, CD30, 4-1BB, OX-40, HVEM/ATAR, and TRANCE-R. Little is known about TRAF1 function except that when overexpressed in transgenic mice TRAF1 plays an inhibitory role in antigen-induced apoptosis of CD8⁺T lymphocytes (Speiser et al., 1997). A biological role for TRAF1 as a regulator of apoptotic signals has been suggested (Lee et al., 1996b). Recently, TRAF1, TRAF2, and the inhibitor-of-apoptosis proteins (IAP) were identified as gene targets of NF- κ B-dependent transcriptional activity (Wang et al., 1998). TRAF1 was in a group of NF- κ B-dependent gene pro-

ducts that function cooperatively at the earliest checkpoint to suppress TNF α -mediated apoptosis. Thus, TRAF1 is one of the key regulators of the cellular stress response.

To gain further insight into the TRAF1 function and to understand the molecular mechanisms responsible for the TRAF1 expression, we have cloned and characterized the murine TRAF1 gene.

2. Materials and methods

2.1. Cloning of the *mTRAF1* cDNA

To obtain the mTRAF1 cDNA we screened the mouse spleen cDNA Lambda ZAPII library (Stratagene) with a 300 bp fragment of the hTRAF1 described earlier (Tsitsikov et al., 1997). This fragment corresponds to the coding region of the hTRAF1 cDNA right 5'-upstream of *HindIII* site. After screening of 0.5×10^6 phage plaques we isolated six positive clones of different sizes.

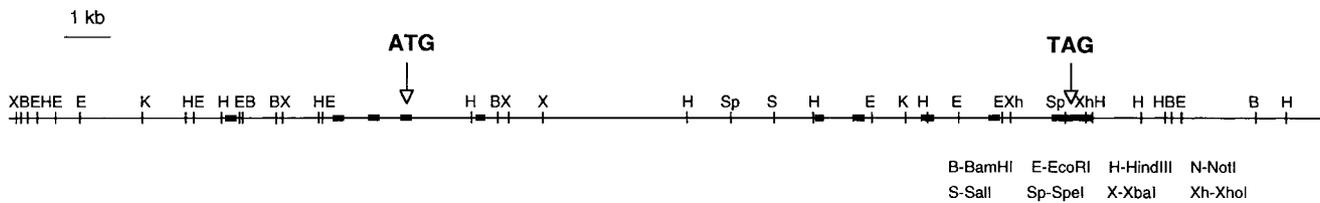
2.2. Cloning and characterization of the *mTRAF1* gene

The mTRAF1 gene was isolated by screening of the Lambda FIXII library (Stratagene) which was made from the liver of strain 129/Sv female mice. We used a 750 bp piece of the murine cDNA located upstream of the *HindII* site as a probe for screening of the genomic library. Screening of approximately 1×10^6 plaques using mTRAF1 cDNA probe yielded 14 different positive phages, which were used for further studies. Restriction mapping was carried out by partial digestion and Southern blotting hybridization with T3 and T7 oligonucleotide probes as suggested by manufacturer. After alignment of individual clones, appropriate DNA fragments were subcloned into pBluescript II KS (+) (Stratagene). Exons were localized by Southern blotting hybridization using synthetic oligonucleotides from different parts of the TRAF1 cDNA and exon/intron junctions were sequenced. The sequencing reactions were performed using Sequenase 2.0 kit (Amersham) or by automatic sequencing in MRRC DNA Sequencing Core facility at Children's Hospital, Boston, MA. The promoter sequence and consensus nucleotide motif analysis were performed using the GENETYX-MAC software (Software Development, Tokyo, Japan).

2.3. Northern blotting analysis

Anti-CD3 (2C11) and anti-CD40 (HM40-3) were purchased from Pharmingen. Anti-mouse IgM (mu chain) F(ab')₂ fragments were purchased from Rockland Inc. LPS (Serotype 0111:B4) and PMA were

A.



B.

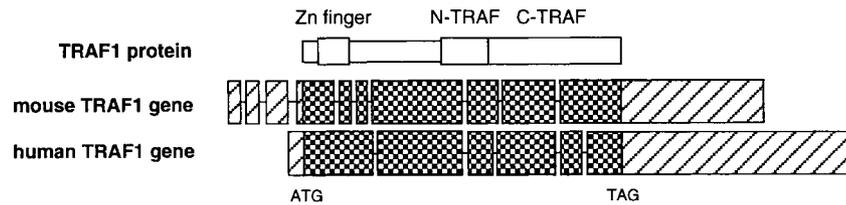


Fig. 1. Genomic organization and restriction map of the murine TRAF1 gene (A). The 10 exons are represented by the closed boxes. Schematic representation of the murine and human TRAF1 genes in comparison with protein structure (B). In the protein, TRAF1 domains are represented by wider boxes and are indicated as zinc finger, N-TRAF and C-TRAF, respectively. In the genes, boxes represent exons and connecting lines represent introns. The coding sequences is represented by shaded areas. The hatched areas indicate untranslated sequences.

purchased from Sigma. Ionomycin (Io) was purchased from Calbiochem. Splenocytes were prepared from the spleen of 2 months old C57BL/6 mice. Isolated cells were maintained in RPMI1640 medium supplemented with 10% FCS and 50 μ M 2-mercaptoethanol and activated overnight with different stimuli including anti-CD3 (2 μ g/ml), anti-IgM (5 μ g/ml), LPS (1 μ g/ml), anti-CD40 (1 μ g/ml) or combination of PMA (20 ng/ml) and Io (1 μ M). Cells of the mouse B cell line M12 were activated overnight with anti-CD40 (1 μ g/ml). Total cellular RNA was prepared with Trisol (Gibco-BRL), fractionated on a formaldehyde-denatured 1% agarose gel and analyzed by Northern blotting with mTRAF1 probe which was used for screening of the genomic DNA clones. We used a 147 bp cDNA probe of the murine constitutively expressed ribosomal protein L32-3A as RNA loading control.

2.4. Identification of the transcription start site

Transcription start site (TSS) was determined by using the Rapid Amplification of cDNA Ends (RACE) system from (Gibco-BRL). Total RNA was prepared from mouse spleen cells activated with anti-CD40 mAb or LPS as described above. First strand cDNA was synthesized by priming with mTRAF1 specific antisense oligonucleotide: GCTCTGACAGGTTCC GCT. DNA corresponding to the 5'-end of the mTRAF1 was amplified by 'anchored' PCR with abridged anchor primer provided by manufacturer and specific antisense oligonucleotide: CCAATTGAAA-

CTC GTTTTCATC. PCR products were subcloned into pCRII vector (Invitrogen) and sequenced.

3. Results and discussion

3.1. Structure of the murine TRAF1 gene

To obtain the mTRAF1 cDNA, we screened of 0.5×10^6 phage plaques and isolated six positive clones of different sizes. The longest insert was 2.3 kb long and the shortest one was 0.9 kb. All of them were analyzed by restriction digestion and partial DNA sequencing. From one of the clones, a 750 bp fragment located upstream of *HindIII* site was used as a mTRAF1 probe for screening of the genomic library.

Screening of approximately 1×10^6 plaques of the genomic library using mTRAF1 cDNA probe yielded 14 different positive phages. After restriction mapping analysis generated with eight different restriction enzymes, *BamHI*, *EcoRI*, *HindIII*, *KpnI*, *Sall*, *SpeI*, *XbaI* and *XhoI*, individual clones were aligned. Hybridization with oligonucleotides corresponding to the most 5'- and 3'-end sequences from mTRAF1 cDNA revealed that full mTRAF1 gene was spread over 18 kb. Exons were localized by Southern blotting hybridization using synthetic oligonucleotides from different parts of the mTRAF1 cDNA. The restriction map and organization of the mTRAF1 gene are illustrated in Fig. 1(B). In order to define the exon-intron junctions, we subcloned different pieces of the phage DNA into pBluescript and used appropriate primers to

Table 1

Splice sites in the murine TRAF1 gene. Sequence of the intron/exon/intron boundaries is shown. The first four and the last four nucleotides of each exon are indicated

5' splice site		3' splice site
TTTTATCCCCATTTTACAG/ATAC	Exon 1	ACAG/GTTGGTTGCCCGTCCCCGC
TCGCAATCCATTTATTTTACAG/ACAT	Exon 2	TGTG/GTGAGTGGGGTGTGGGAAGG
GTCCTCTGCTCTACTTAACA/ACCT	Exon 3	AAAG/GTGAGCCCCATTGTTCCAAA
ACTTATTCGTGTTTCCCCAG/AGAT	Exon 4	TGAG/GTGAGGGGACAAAGCCAAGG
GACACAGTTATCATTTCCAG/GTTC	Exon 5	GAAG/GTATGTTAGCACAGACTAGA
TAAAGGCCCTCACTCTGTCA/GGGA	Exon 6	CAAG/GTAAGAAATTGGTAATGNCA
CTGACCAGCCTTCCCTACAG/GTGG	Exon 7	GAGG/GTGAGTGAGATGGGCATGTT
ACACTGGCTTACCTTGCAG/CTTT	Exon 8	CCAG/GTGACTCCTCCTCGCGGCCA
TTCCTGCCTTACCCTACAG/GTCA	Exon 9	CAAG/GATGGGGAGAAGATGGTTTG
	Exon 10	

sequence from exon into intron down to known restriction enzyme sites and back through exon. The results showed that the mTRAF1 gene consists of 10 exons. By comparison the human TRAF1 gene was found to consist of 6 exons comprising approximately 12 kb. The position of the introns was determined by comparison with the human TRAF1 gene and following the GT/AG splice rule (Mount, 1982). The exon-intron boundaries and the corresponding splice sequences are shown in Table 1. The nucleotide sequences for the exons of the mTRAF1 gene in the 129 Sv mouse strain was in agreement with the published mTRAF1 cDNA sequence (Rothe et al., 1994).

In contrast to human TRAF1 mRNA, the murine TRAF1 mRNA has a very long 5'-UT region which is encoded by three separate exons (Mosialos et al., 1995; Rothe et al., 1994). The fourth exon encodes for the rest of the 5'-UT end and for the first 40 amino acids (a.a.), including a part of the zinc-finger domain. Exon 5 encodes for a.a. 41–68, including the second part of the zinc-finger domain. Exon 6 encodes for a.a. 69–91. Exon 7 encodes for a.a. 92–228 and exon 8 encodes a.a. 229–287, respectively. These two exons contain the N-TRAF domain. Exon 9 encodes for a.a. 288–337, including a part of the C-TRAF domain. Exon 10 is the largest one and encodes for a.a. 338–409, including the rest of the C-TRAF domain and 3'-UT region.

The human TRAF1 gene was recently cloned (Siemiński et al., 1997). A comparison of the architecture of the murine and the human TRAF1 genes is presented in Fig. 1(B). While murine and human TRAF1 proteins share a high degree of primary sequence homology (86%), the murine and human TRAF1 genes have only two conserved exon-intron splicing sites between exons 7–8 and 9–10, which corresponds to sites between exons 2–3 and 4–5 for human. All other exon-intron splicing sites are different between human and murine counterparts. These observations suggest divergent derivation of the murine and the human TRAF1 genes from a common

ancestor gene, probably featuring different mechanisms of regulation of expression.

3.2. Transcription start sites and promoter region of the mTRAF1 gene

To determine TSS for the mTRAF1 gene we purified total RNA from mouse spleen cells activated with anti-CD40 mAb or LPS and employed the RACE system as described in Materials and Methods section. We have sequenced more than 12 DNA fragments and all of them have varying lengths of the 5' end. The four longest fragments started at positions –42, –19, –15 and +4, relative to the 5' end of the published sequence of the mTRAF1 cDNA. These data indicated that mTRAF1 mRNA transcription is probably initiated at multiple positions and has several TSS. Therefore we postulate the 5' end of the published sequence corresponding to the mTRAF1 cDNA as a

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-260 AAGCTTGCTG TTTTCCACT CTTGCCTTGC GGGCACTTCA GGTGGAGCT
-210 CAATGTTGTT TGTGTGTGTG CTGAGTATCT CAGGAGTGCA AGCCACTGAT
-160 GGAATGTTAG GAGTCAGCTA GCACCAGGAC CCCAACAGAA GCACTCCAGT
-110 TCTGTTTCCT TGAGGGAGGA GCGATCAGCC CGCCGCCCGG CCAGCCTCCG
-60 AAGCCAGCCC AGCTCAGTCC* CACCCGCCTG CCAGCCCAG CTCCCTTG
-10 CTTCCGCAG CCAGCCCGG TTTCTGTGCC CAAGGACGCT ACCGCCCGGT
40 GCGAGCAGAA GGCGGCGCAC AGGTTGGTTG CCCGTCCCCC GCGGGTCCCC
90 AGGGTCCCCA AGTGCACCA TACTCAGCCT

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Fig. 2. Nucleotide sequence of the murine TRAF1 promoter region. The numbering indicates the first nucleotide position relative to the 5'-end of the published cDNA sequence. This position was assumed to be a transcription start site, designated as +1 and indicated by arrow. The position of the 5'-ends of the 5'-RACE products are indicated by *. The first exon is underlined. The GC-stretch, three repeats of CCAGCCCAGC and three repeats of GTCCCC are underlined.

TSS. The nucleotide sequence of -240 and $+119$ bp of TSS was determined (Fig. 2).

The 5'-flanking region of the mTRAF1 gene was analyzed for the presence of sequence motifs, which could be responsible for the regulation of TRAF1 gene transcription. We found that the mTRAF1 promoter lacks TATA- or CAAT-like sequences but that it has a GC-rich (75%) region located at positions -82 to $+100$ relative to TSS. Within this region, we found a 15 bp-long GC-stretch (-82 to -68) which might be a potential Sp-1-binding site. Also within this GC-rich region, we found three identical 10 bp long repeats (CCAGCCCAGC) which were separated by 21 and 15 bp. At the 3' downstream sequence relatively to TSS, we also found three 6 bp repeats (GTCCCC) which were separated by 5 and 3 bp. Computer analysis for all these repeats revealed no matching transcriptional factor binding sites. Thus, the absence of TATA or CAAT boxes, the presence of a GC-rich region and the identification of several transcription initiation sites for mTRAF1 suggest that it is probably an Sp-1-dependent promoter. Further analysis is necessary to characterize the mTRAF1 gene promoter and to understand the underlying molecular mechanisms of the transcriptional regulation of mTRAF1 expression.

As mentioned above, the murine TRAF1 mRNA has a longer 5'-UT end than its human counterpart. Furthermore, while the corresponding part of the murine TRAF1 is encoded by four separate exons with one of them containing the coding sequence, the human TRAF1 mRNA 5'-untranslated region end is encoded by one exon, which contains a part of the coding sequence. These differences in the gene structure suggest that the murine and the human TRAF1 promoters might have completely different promoter sequences. Indeed the human TRAF1 5'-upstream region does not possess any TATA or CAAT boxes, like the murine TRAF1 promoter. But in contrast to the murine TRAF1 promoter, the human TRAF1 5'-upstream region does not contain a GC-rich sequence. Analysis of the human TRAF1 promoter revealed that it contains two overlapping ~ 44 bp long repeats at positions -194 to -152 and -155 – 113 . There is also a poly-A stretch at positions -223 to -210 . One may speculate that there may be a retroposed pseudogene in front of the human TRAF1 gene. We could not find such homologous repeats in the murine TRAF1 promoter. Taken together these observations suggest that the murine and the human TRAF1 gene might well have different ways of transcriptional regulation of their expression in these species and further analysis will be needed to understand the molecular mechanisms for their transcription.

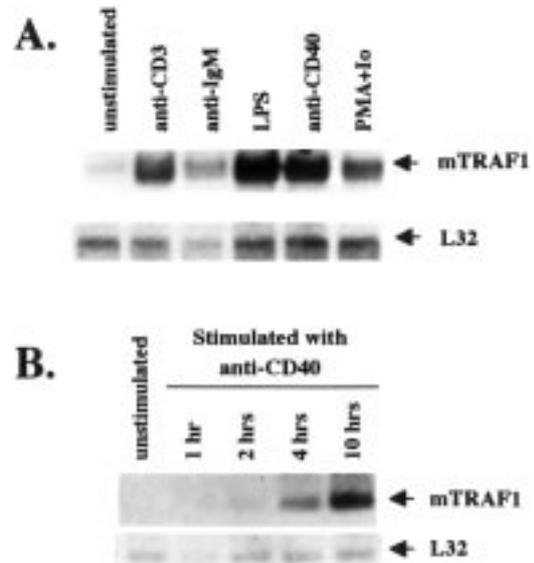


Fig. 3. Regulation of TRAF1 gene expression in activated lymphocytes. TRAF1 gene expression was detected by Northern blotting analysis. The top panels represent autoradiograph with a mTRAF1 cDNA probe. The bottom panels represent autoradiograph with a loading control of L32 cDNA probe. (A) Mouse spleen cells were left unstimulated or stimulated overnight with different stimuli including anti-CD3 (2 $\mu\text{g/ml}$), anti-IgM (5 $\mu\text{g/ml}$), LPS (1 $\mu\text{g/ml}$), anti-CD40 (1 $\mu\text{g/ml}$) or combination of PMA (20 ng/ml) and Io (1 μM). (B) M12 cells were left unstimulated or stimulated with anti-CD40 (1 $\mu\text{g/ml}$). Cells were harvested at indicated time.

3.3. TRAF1 mRNA is expressed in activated lymphocytes

It was shown that TRAF1 expression is tissue-specific: the murine TRAF1 mRNA could only be detected in spleen, lung, and testis (Mosialos et al., 1995; Rothe et al., 1994). To examine TRAF1 expression in activated lymphocytes, we performed Northern blot analyses with total RNA from purified spleen cells activated with different stimuli including anti-CD3, anti-IgM, LPS, anti-CD40 antibodies and a combination of PMA and ionomycin. Fig. 3(A) shows that while TRAF1 mRNA is hardly detectable in unstimulated lymphocytes, it is strongly upregulated upon activation. Moreover, T cell-specific activation (anti-CD3), B cell-specific activation (anti-IgM, LPS and anti-CD40), or T and B lymphocyte activation, that bypasses cell surface receptor signaling (PMA+Io), all were able to activate TRAF1 expression in spleen cells. These results demonstrate that TRAF1 mRNA is inducible in both T and B lymphocytes and suggests that TRAF1 is important for further lymphocyte survival and function.

To determine the kinetics of TRAF1 mRNA up-regulation after activation in B cells, we examined the effect of the anti-CD40 antibody on TRAF1 mRNA expression in a B cell line, M12. Cells were activated

and harvested at 0, 1, 2, 4 and 10 h and analyzed for the mTRAF1 mRNA expression by Northern blotting. As shown in Fig. 3(B), TRAF1 mRNA was not detectable in non-stimulated cells (lane 1) but was detectable after 2 h of activation (lane 2) and significantly increased after 10 h (lane 5). Analysis of the constitutively expressed L32 mRNA showed comparable amount of expression in all wells. These results suggest that TRAF1 gene belongs to a class of early expression genes which may be necessary for cell activation and survival. Originally TRAF1 was cloned from the murine IL-2-dependent T cell line CT6 (Rothe et al., 1994). In our laboratory, we have isolated TRAF1 from the IL-6-dependent cell line KT-3 while we did not detect it in a Jurkat cell line, which does not require the presence of IL-6 for growth (Tsitsikov et al., 1997). In humans, TRAF1 was described as a protein induced by EBV infection (Liebowitz, 1998; Mosialos et al., 1995). hTRAF1 mRNA was at least 8 fold more abundant in the EBV-infected BL41/B95-8 or IB4 cells than in non-infected BL41 cells. Particularly, LMP1 plays a central part in this process by mimicking members of the TNFR family and induces the expression of TRAF1 along with many other proteins in EBV-negative Burkitt lymphoma BL41 cells, thereby transmitting growth signals from the cell membrane to the nucleus of the transformed cell. Moreover, TRAF1 proved to be absent from all resting lymphocytes as well as from macrophages and accessory cells and present in a few perifollicular and intrafollicular lymphoid blasts (Durkop et al., 1999). In contrast, there was a high and consistent TRAF1 expression in EBV-induced lymphoproliferations and Hodgkin's disease. Taken together, these data support the idea that TRAF1 expressed in activated lymphocytes and its expression may be necessary for the regulation of signal transduction by the members of TNFR superfamily, including CD30, 4-1BB, OX40, HVEM/ATAR or TRANCE-R.

Acknowledgements

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