A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40

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Abstract CD40 is a member of the tumor necrosis factor receptor (TNF-R) family that regulates B-lymphocyte proliferation, immunoglobulin class-switching, and apoptosis through poorly defined signal transduction mechanisms. Using a yeast two-hybrid method, cDNAs were obtained that encode a novel protein, CD40-associated protein-1 (CAP-1), which binds specifically to the cytosolic domain of CD40 but not TNF-R1, TNF-R2, or Fas. The CAP-1 protein contains a C-terminal domain that shares strong amino acid sequence homology with a unique domain found recently in two putative signal transducing proteins that bind to the TNF-R2 cytosolic tail, TRAF1 and TRAF2. This C-terminal region of CAP-1 was sufficient to mediate binding to CD40 and homodimerization of CAP-1 proteins. The N-terminal portion of CAP-1 contains a RING finger motif and three zinc finger-like domains similar to those found in several regulatory proteins that interact with DNA or RNA. CAP-1 thus represents a new member of a family of potential signal transducing proteins that contain a conserved domain (the TRAF domain), bind to the cytosolic regions of particular members of TNF-R family proteins, and that can form homo- and heterotypic dimers.

Key words: CD40; TRAF; Signal transduction

1. Introduction

CD40 is a ~50-kDa glycoprotein receptor that is expressed on the surface of B-lymphocytes, some activated T-cells, monocytes, follicular dendritic cells, basal epithelial cells as well as some epithelial and non-epithelial carcinomas. The functions of this receptor have been most extensively studied in B-cells, where it is believed to play an important role in regulation of proliferation and apoptosis, immunoglobulin class-switching, generation of memory B-cells, modulation of cytokine gene expression, and cell adhesion (reviewed in [1–3]). The ligand for CD40 is expressed on the surface of activated helper T-cells, as well as mast cells and basophils, as a type-II transmembrane glycoprotein [4–6]. CD40-L thus triggers CD40 through a mechanism involving cell–cell contact. The importance of the CD40/CD40-L interaction for the regulation of T-cell-dependent humoral immune responses has been demonstrated by experiments where antibodies to CD40-L were shown to inhibit the generation of primary and secondary humoral immune responses [7]. Moreover, mutations in the CD40-L in humans cause an X-linked immunodeficiency syndrome characterized by excess production of IgM and absence of production of IgG, IgA, and IgE caused by a failure of B-cells to undergo Ig class-switching [8–10]. Thus, CD40-L/CD40 interactions appear to be essential for Ig class switching in vivo. Likewise, transgenic mice with homozygous disruptions of their CD40 genes fail to form germinal centers and do not produce IgG, IgA, and IgE response to thymus-dependent antigens but can mount normal IgM and IgG responses to T-independent antigens [11]. Antibodies to CD40-L prevent collagen-induce arthritis in animal model [12], suggesting that inhibition of CD40-triggering could provide a means of therapeutically intervening in some types of autoimmune dieases. The expression of CD40-L on mast cells and basophils has also been reported to mediate induction of IgE synthesis by B-cells when co-stimulated with IL-4 [6], implying an important role for CD40-L/CD40 interactions in atopic diseases.

CD40 and its ligand are members of the Tumor Necrosis Factor Receptor (TNF-R) family and TNF-family, respectively. The TNF-R family includes the p50/55-TNF-R1, p75/80-TNF-R2, p75-Nerve Growth Factor (NGF) Receptor, CD27, CD30, Fas, among others [13]. The predicted cytoplasmic domain of CD40 contains just 62 amino-acids and bears no resemblance to kinases or other enzymes that might suggest a mechanism by which this protein transduces signals into cells [14]. A region within the cytoplasmic domain of CD40 however has limited homology to a conserved domain found in the cytosolic tails of p75-NGF-R (22%), TNF-R1(31%), and Fas (41%). In TNF-R1 and Fas, this conserved domain is required for induction of signals resulting in cell death [15,16]. Interestingly, though best known for their roles in generating signals that suppress apoptosis, both CD40 and p75-NGF-R have been shown to paradoxically accelerate apoptosis when ectopically expressed in certain neural cell lines in the absence of ligands [17,18]. In these same cells, CD40 and p75-NGF-R can also delay cell death relative to untransfected cells when triggered with appropriate ligands. Thus, it is possible that CD40 and NGF-R (like TNF-R and Fas) can regulate signal transduction pathways leading to cell death under some circumstances. To gain further insights into the mechanisms by which CD40 transduces signals into cells, we used a yeast two-hybrid approach to clone cDNAs that encode proteins capable of specifically binding to the cytosolic domain of this receptor. A novel protein was thus discovered which we have termed CAP-1 for CD40-associated protein-1.

2. Materials and methods

2.1. Plasmid constructions

The cDNA encoding various fragments of CD40 were generated by PCR from the plasmid pCDM8-CD40 (gift of B. Seed) [14] using the following forward (F) and reverse (R) primers containing *Eco*RI (underlined) and *Bcl*I (italic) sites (bold indicates stop codon): CD40 (216–277), 5′-GGAATTCAAAAAGGTGGCCAAG-3′ (F1) and 5′-*TGATCA*TCACTGTCTCTCCTGCAC-3′(R1); and CD40 (225–269),

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5′-GGAATTCAAGGCCCCCCACCCCAAG-3′ (F2) and 5′-*TG-ATC*AACTCTCTTTGCCATCCTC-3′ (R2). The PCR products were digested with *Eco*RI and *Bcl*I, then directly cloned into the *Eco*RI and *Bam*HI sites of the yeast two hybrid plasmid pBTM 116 (gift of S. Hollenberg, U. Washington; Seattle) in frame with the upstream LexA DNA-binding domain sequences [19]. For expression of LexA-Fas and LexA-TNF-R2 fusion proteins in yeast, the cytosolic domains of Fas and TNF-R2 were amplified by PCR from the plasmids pBS-APO14.1 (gift of P. Krammer) and pBluescript-p80-TNF-R2 (gift of C. Smith, Immunex, Inc., Seattle, WA), respectively, using the primers 5′-GGAATTCAAGAGAAAGGAAGTACA-3′(forward/Fas) and 5′-*GTGA*TCACTAGACCAAGCTTTGGAT-3′ (reverse/Fas) and 5′-GGAATTCAAAAAGAAGCCCTTGTGCCT-3′ (forward/TNF-R2) and 5′-G*GTCGAC*TTAACTGGGCTTCATCCCA-3′(reverse/TNF-R2). The Fas PCR product was digested with *Eco*RI and *Bcl*I and cloned into *Eco*RI/*Bam*HI-digested pBTM-116, whereas the TNF-R2 PCR product was digested with *Eco*RI and *Sal*I and cloned into *Eco*RI/*Xho*I-digested pBTM 116. Additional pBTM 116 plasmids encoding portions of the Bcl-2, Ha-Ras (V12), and Lamin-C proteins have been described [19,20].

For expression as GST-fusion proteins in *E. coli*, the pBTM 116-CD40 (225–269), Fas, and TNF-R2 plasmids were digested with *Eco*RI and either *Xho*I or *Sal*I and the inserts subcloned in-frame with the upstream GST sequences in pGEX-4T-1. For GST-CD40(220–277), a *Bal*I fragment encoding amino-acids 220–277 was excised from pCDM8-CD40 and subcloned initially into the *Hin*cII site of Bluescript pSKII (Stratagene, Inc., La Jolla, CA). The pSKII-CD40/*Bal*I plasmid was then cut with *Xho*I and *Eco*RI and the CD40 sequences subcloned into a modified verion of pGEX-2T-1 in frame with the GST sequences. For GST-TNF-R1, the cytoplasmic domain of TNF-R1 was PCR amplified from the plasmid pUC19-p55-TNF-R1 (gift of W. Lesslauer; Hoffmann La-Roche Inc., Basal, Switzerland) using the primers 5′-G*GGATCC*GCTACCAACGGTGGAAG-3′ (forward/TNF-R1) and 5′-G*GTCGAC*TCATCTGAGAAGACTGGG-3′ (reverse/TNF-R1), digested with *Bam*HI and *Sal*I and subcloned into pGEX-5X-1. For GST-CAP-1, the pACT-2229 cDNA was digested with *Bgl*II, and the CAP-1 cDNA (363–543) was subcloned into pGEX-3X.

2.2. Two-hybrid assays

Library screening by the yeast two-hybrid methods was performed as described [19,21] using a human B-cell library in lambda-ACT (gift of S. Elledge, Baylor; Houston, TX) and L40 strain *S. cerevisiae* [*MATa, trp1, leu2, his3, ade2, LYS2::(lexAop)*^{4}*-HIS3, URA3::(lexAop)*^{8}*-lacZ*]. Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone, and 2% glucose, or in Burkholder's minimal medium fortified with appropriate amino-acids as described previously [20]. Transformations were performed by a LiCl method using 0.5 mg of pACT-cDNA library DNA, 1 mg of denatured salmon sperm carrier DNA, and ~5×10^9 L40-strain cells. The mating strain was NA87–11A (*MATα, leu2, his3, trp1, pho3, pho5*) which had been transformed with pBTM116 plasmids producing CD40 cytoplasmic domain or various control proteins and selected on tryptophan-minus media. To confirm the specificity of interactions, 5 μg of various pBTM116 plasmids and 5 μg of either pACT-121 or pACT-2229 were transformed into L40 cells and plated on semi-solid media lacking tryptophan and leucine. Colonies were then transfered to histidine-deficient plates or histidine-containing media with X-gal. β-Galactosidase plate assays were performed ~12 h later and filter assays hrs later as described previously [20].

2.3. In vitro protein-binding assays

The CAP-1 cDNA was PCR amplified using a forward primer containing a T7 promoter (5′-TAATACGACTCACTATAGGGAGAC-CACATGGATGATGTATATAACTATCATTTC-3′) and a reverse primer (5′-CTACCAGAATTCGGCATGCCGGTAGAGGTGTGG-TCA-3′) from 1 μg of pACT-2229 plasmid DNA. One-tenth of the resulting PCR product (~0.1 μg) was then directly transcribed and translated in vitro in the presence of [^{35}S]methionine using 12.5 μl of reticulocyte lysate (TNT-Lysate; Promega, Inc.; Madison, WI). Glutathione-Sepharose bead containing 5 μg of GST fusion proteins were incubated with 10 μl of in vitro translated protein in 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM DTT, 2 mM EDTA, 0.1% NP-40, 1 mM PMSF, and 1 μg/ml leupeptin for 16 h at 4°C. The beads were washed vigorously 5× in the same solution, pelleted by centrifugation, and boiled in Laemmli sample buffer before analysis by SDS-PAGE and fluorography.

For experiments with cell lysates, 2×10^7 RS11846 B-cell lymphoma cells were lysed in 0.25 ml of ice-cold 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, with protease inhibitors (PMSF, aprotinin, leupeptin, benzamidine, and pepstatin) and nuclei and debris were removed by centrifugation as described. The resulting supernatants were then incubated with 5 μg of affinity-purified GST-CAP-1 (363–543) or GST-control protein immobilized on 50 μl of glutathione-Sepharose beads for ~16 h at 4°C, then washed × 10 in 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM PMSF, 1 μg/ml leupeptin. Proteins adsorbed to beads were then boiled in Laemmli sample buffer and analyzed by SDS-PAGE (12% gels) and transfered to nitrocellulose. The resulting blots were preblocked in Tris-buffered saline (pH 7.4) containing 5% non-fat milk and 3% bovine serum albumin (BSA) and then incubated with 2 μl/ml of anti-human CD40 monoclonal antibody B-B20 (Biosource, Inc.; Camarillo, CA) followed by horseradish peroxidase-conjugated goat anti-mouse IgG and detection using an ECL method (Amersham, Inc.).

2.4. Screening of cDNA libraries and RT-PCR

The insert from pACT-2229 was excised with *Xho*I, ^{32}P-labeled by a random primer method, and used to screen a human fetal brain cDNA library (Clonetech, Inc.) in λ-gt11. Two cDNA clones were obtained, PCR amplified using primers flanking the cloning site in λ-gt11, and the resulting PCR products were subcloned into a *Eco*RV-digested, T-tailed pSK-II plasmid and their DNA sequence determined by the dideoxynucleotide method.

For 5′-RACE, 10 μg of total RNA from Raji B-cell lymphoma cells was reverse-transcribed using a specific primer 5′-GCGTTAACTGC-TCTGCACAA-3′ and recombinant Moloney murine leukemia virus reverse transcriptase (Gibco/BRL, Inc.; Gaitherburg, MD). The resulting cDNA was subjected to homopolymeric tailing using dCTP and terminal deoxynucleotidyl transferase using a kit from BRL/GIBCO, and then PCR amplified using a universal 5′-anchor primer provided by the manufacturer and a CAP-1-specific reverse primer 5′-GTACA-TTTTGGACTTGAAGA-3′. The final PCR product was subcloned into T- tailed pSK-II and sequenced.

3. Results

3.1. Cloning of cDNAs encoding a CD40-binding protein by yeast two-hybrid method

A yeast two hybrid approach was employed to attempt to clone cDNAs encoding proteins capable of binding to the cytoplasmic domain of CD40. For these experiments, the entire cytoplasmic tail of CD40 (amino-acids 216–277) was expressed in yeast as a fusion protein with an NH_2-terminal LexA DNA binding domain. Yeast were then transformed with a human B-cell cDNA library in an plasmid that expresses random cDNAs as fusion proteins with a Gal4 *trans*-activation domain. Positive interactions between the LexA-CD40 bait protein and Gal-4-fusion proteins were detected using either a *HIS3* gene or *lacZ* gene under the control of *lexA* operators. From an initial screen of ~3×10^7 transformants, 2,640 clones were identified that *trans*-activated the *HIS3* reporter gene based on ability to grow on histidine-deficient media. Of these, 166 were also produced β-galactosidase. These 166 candidate transformants were then cured of the LexA/CD40 bait plasmid by growth in media containing tryptophan and then mated with each of 4 different indicator strains of cells containing one of the following LexA bait proteins: cytosolic domain of CD40 (amino-acids 216–277), cytoplasmic domain of Fas (191–335), Bcl-2 (83–218), or Lamin-C. This resulted in just two clones that grew on histidine-deficient media and produced β-galactosidase when expressed in combination with the LexA-CD40 bait protein but not the other 3 control LexA fusion proteins.

DNA sequencing demonstrated that these represented identical cDNAs, presumably arising during library amplification. An open reading frame of 543 bp (181 amino-acids) followed by a stop codon and a ~457 bp 3′-untranslated region was identified fused with the upstream Gal4 *trans*-activation domain sequences (hereafter termed CAP-1).

3.2. *CAP-1 interacts with a subregion of the CD40 cytoplasmic domain that is conserved in some other members of the TNF receptor family*

The Gal4/CAP-1 plasmids identified through the cDNA library screening experiment described above were isolated and retransformed into yeast cells with various LexA fusion proteins to confirm their ability to bind to the cytoplasmic domain of CD40 in two-hybrid assays. As summarized in Table 1, the CAP-1 protein fragment interacted with LexA fusion proteins containing not only the complete cytosolic domain of CD40 (amino-acids 216–277), but also a smaller portion of this domain (amino-acids 225–269) that has homology to a conserved domain found in the cytosolic domains of TNF-R1, Fas, and the p75 subunit of the NGF receptor [22]. CAP-1, however, failed to interact with the cytoplasmic domains of Fas and TNF-R2 in these two-hybrid experiments, as well as a variety of other proteins, including Ha-Ras, Bcl-2, and Lamin-C.

3.3. *Interactions of CAP-1 with CD40 cytoplasmic domain demonstrated by in vitro binding assays*

To confirm the interaction of CAP-1 with the cytoplasmic domain of CD40, in vitro binding assays were performed using in vitro translated CAP-1 and affinity-purified GST-CD40 fusion proteins. For these experiments, the CAP-1 cDNAs identified by library screening were transcribed and translated in vitro using rabbit reticulocyte lysates in the presence of [^{35}S]L-methionine. The resulting ^{35}S-labeled CAP-1 protein was then incubated with various GST-fusion protein, as indicated in Fig. 1A. Using this in vitro binding assay, CAP-1 was shown to interact specifically with GST-fusion proteins containing the nearly full-length CD40 cytoplasmic domain (220–277) as well as the conserved subregion of the CD40 cytosolic domain (225–269). No interactions were detected in vitro between ^{35}S-labeled CAP-1 and GST-fusion protein containing the cytosolic domains of Fas, TNF-R1, or TNF-R2 (Fig. 1A). CAP-1 also did not interact with a GST non-fusion protein but did interestingly

Table 1
Specific interaction of CAP-1 with CD40 cytoplasmic domain

pBMT116 (LexA)	pACT (Gal4)	His	β-Gal
CD40 (216–277)	2229	+	+
CD40 (225–269)		+	+
TNFR2 (288–461)		–	–
Fas (191–335)		–	–
Ras (V12)		–	–
Bcl-2 (83–218)		–	–
Lamin-C		–	–

pBTM116 plasmids producing LexA DNA-binding domain fusion proteins (listed at left) were co-transformed with pACT-CAP-1 (clone 2229) which encodes the C-terminal 181 amino-acids of CAP-1 fused to a Gal-4 *trans*-activation domain (5 μg each) into L40 stain yeast. Transformed cells were grown on semi-solid media lacking histidine or containing histidine as a control. Plasmid combinations that resulted on growth on histidine-deficient media were scored as positive (+). β-galactosidase activity of each colony was tested by filter assay and scored as blue (+) versus white (–).

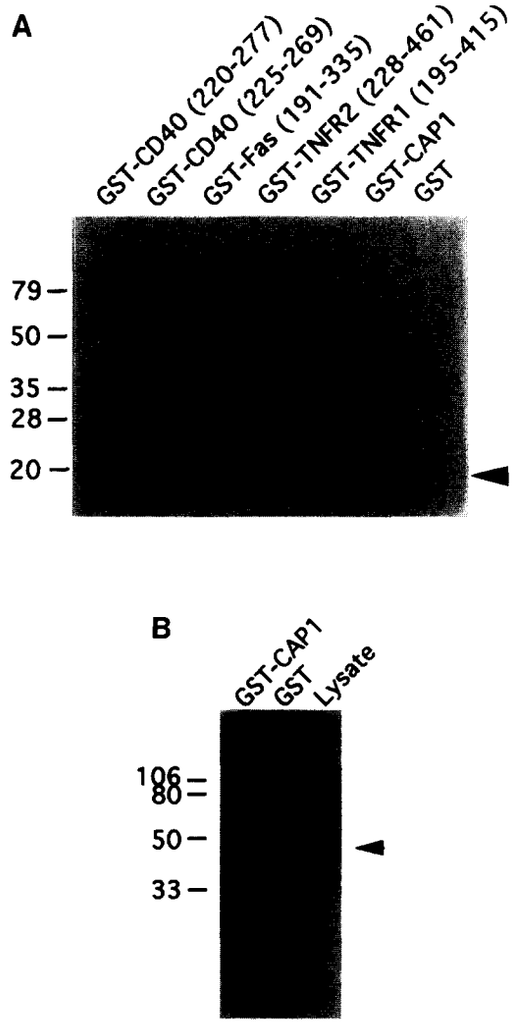

Fig. 1. In vitro binding of CAP-1 to CD40 cytoplasmic domain and CAP-1 C-terminal domain. In (A), various GST-fusion proteins as indicated were affinity-purified on glutathione-Sepharose. Beads containing ~5 μg of GST-proteins were incubated with ^{35}S-L-methionine-labeled in vitro translated CAP-1 (C-terminal 181 amino-acids). After extensive washing, beads were boiled in Laemmli buffer and the eluted proteins were subjected to SDS-PAGE followed by fluorographic detection of ^{35}S-CAP-1 protein (bold arrow) which migrated as a doublet here, presumably due to translation from both the first AUG of Gal-4 in pACT-2229 as well as an internal start codon located either 4 or 15 residues downstream (from multiple cloning site of pACT). In (B), GST-CAP-1 (C-terminal 181 amino-acids) and GST control proteins were immobilized on glutathione-Sepharose and incubated with detergent lysates prepared from RS11846 B-cell lymphoma cells. Beads were washed extensively, and associated proteins were eluted by boiling in Laemmli buffer followed by SDS-PAGE and transfer to nitrocellulose filters. RS11846 cell lysates were also run directly in the gel as a positive control (far right lane). The resulting blot was incubated with anti-CD40 monoclonal antibody B-B20 , which was detected subequently by an ECL method using 10 min exposed to X-ray film. The position of CD40 is indicated by the bold arrow.

interact with GST-CAP-1, implying that CAP-1 can homodimerize. The specificity of these results was further confirmed by experiments where a variety of other ^{35}S-labeled in vitro translated proteins including Bcl-2, Bax, Lyn were tested and found not to bind to GST-CD40 fusion proteins under the same conditions used here of ^{35}S-CAP-1 (not shown).

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CAP-1      MESSKKMDSP GALQTNPPLK LHTDRSAGTP VFVPEQGGYK EKFVKTVEDK   50
TRAF2      MAAA-SVTSP GSLELLQP-G FSKTLLGTRL EAKYLCSACK NILRRPFQAQ   48
TRAF1      MASS-S--AP DENE-FQF-G CPPAPCQDPS EPRVLC--CT ACLSENLRDD   43
Consensus  MASS-S..SP G.LE..QP-G .......... E...LC..CK ..L.....D.   50

CAP-1      YKCEKCHLVL CSPKQTECGH RFCESCMAAL LSSSSPKCTA CQESIVKDKV  100
TRAF2      CGHRYCS--F CLTSILSSGP QNCAACVYEG LYEEG---IS ILESSS---A   90
TRAF1      -EDRICP--K CRADNLHPVS PGSP--LTQE KVHSD---VA EAEIMC---P   82
Consensus  ...R.C.--. C....L..G. ..C..C.... L..S.---.A ..ES..---.  100

CAP-1      FKDNCCKREI LALQIYCRNE SRGCAEQLTL GHLLVHLKND CHFEELPCVR  150
TRAF2      FPDNAARREV ESLPAVCPND --GCTWKGTL KEYESCHEGL CPF--LLTEC  136
TRAF1      FAG------- ----VGCSFK --GSP--QSM QEHEATSQSS HLY--LL---  112
Consensus  F.DN...RE. ..L...C.N. --GC....TL .E.E..... C.F--LL...  150

CAP-1      PDCKEKVLRK DLRDHVEKAC KYREATCSHC KSQVPMIALQ KHEDTDCPCV  200
TRAF2      PACKGLVRLS EKEHHTEQEC PKRSLSCQHC RAPCSHVDLE VHYEV-CPKF  185
TRAF1      -----LAVL- -KEW---KSS PGSNLG---- SAP---MALE RNL----SEL  141
Consensus  P.CK.LV.L. .KE.H.EK.C P.R.L.C.HC .AP....ALE .H...-CP..  200

CAP-1      VVSCPHKCSV QTLLRSEGTN QQIKAHEASS AVQHVNLLKE WSNSLEKKVS  250
TRAF2      PLTC-DGCGK KKIPR-ETFQ DHVRACSKCR VLCRFHTVG- -CSEMVETEN  231
TRAF1      QL-------- -QAAV-EATG DLEVDC--YR APC------- -C------ES  165
Consensus  .L.C-..C.. ....R-E.T. D...AC...R A.C.....- -C.....ES  250

CAP-1      LLQNESVEKN KSIQSLHNQI CSFEIEIERQ KEMLRNNESK ILHLQRVIDS  300
TRAF2      -LQDHELQRL REHLAL--LL SSF-LEAQAS PGTLNQVGPE LLQRCQILE-  276
TRAF1      -QEELALQHL ---------- ---------- ------VKEK LLAQ---LE-  184
Consensus  -LQ...LQ.L .....L--.. .SF-.E.... ...L..V..K LL.....LE-  300

CAP-1      QAEKLKELDK EIRSFRQNWE EADSMKSSVE SLQNRVTELE SVDKSAGQVA  350
TRAF2      --QKIATFEN IVCVLNRE-V ERVAVTAEAC SRQHRL-DQD KIEA------  316
TRAF1      --EKLRVFAN IVAVLNKE-V EASHLALAAS IHQSQL-DRE HLLS------  224
Consensus  --EKL..F.N IV.VLN.E-V EA......A. S.Q.RL-D.E ....------  350

CAP-1      RNTGLLESQL SRHDQMLSVH DIRLADMDLR FQVLETASYN GVLIWKIRDY  400
TRAF2      -----LSNKV QQLERSIGLK DLAMADLEQK VSELEVSTYD GVFIWKISDF  361
TRAF1      -----LEQRV VELQQTLAQK DQVLGKLEHS LRLMEEASFD GTFLWKITNV  269
Consensus  -----LE..V ..L.Q.L..K D..LADLE.. ...LE.ASYD GVFIWKI.D.  400

CAP-1      KRRKQEAVMG KTLSLYSQPF YTGYFGYKMC ARVYLNGDGM GKGTHLSLFF  450
TRAF2      TRKRQEAVAG RTPAIFSPAF YTSRYGYKMC LRVYLNGDGT GRGTHLSLFF  411
TRAF1      TKRCHESVCG RTVSLFSPAF YTAKYGYKLC LRLYLNGDGS GKKTHLSLFI  319
Consensus  TRR.QEAV.G RT.SLFSPAF YT..YGYKMC LRVYLNGDG. GKGTHLSLFF  450

CAP-1      VIMRGEYDAL LPWPFKQKVT LMLMDQGSSR RHLGDAFKPD PNSSSFKKPT  500
TRAF2      VVMKGPNDAL LQWPFNQKVT LMLLDH-NNR EHVIDAFRPD VTSSSFQRPV  460
TRAF1      VIMRGEYDAL LPWPFRNKVT FMLLDQ-NNR EHAIDAFRPD LSSASFQRPQ  368
Consensus  VIMRGEYDAL LPWPF.QKVT LMLLDQ-NNR EH.IDAFRPD ..SSSFQRP.  500

CAP-1      GEMNIASGCP VFVAQTVLE- -NGTYIKDDT IFIKVIVDTS DLPDP       543
TRAF2      SDMNIASGCP LFCPVSKME- AKNSYVRDDA IFIKAIVD-- -LTGL       501
TRAF1      SETNVASGCP LFFPLSKLQS PKHAYVKDDT MFLKCIVD-- --TSA       409
Consensus  SEMNIASGCP LF.P.SKLE- .K..YVKDDT IFIK.IVD-- -LT..       545
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Fig. 2. Alignment of predicted amino-acids sequences of CAP-1 with TRAF1 and TRAF2. An alignment of the predicted amino-acids sequences of the human CAP-1, TRAF2, and TRAF1 proteins is shown. Residues that are identical in all three proteins are shown in bold type. A concensus sequence is provided below in italics, with residues that are shared among two of the three proteins in plain type and residues that are identical in all three proteins in bold type.

As another approach to examining the interaction of CAP-1 with CD40, in vitro binding experiments were performed using cell lysates derived from an CD40-expressing human B-cell lymphoma cell line RS11846. GST control protein or GST-CAP-1 fusion protein were immobilized on glutathione-Sepharose beads and mixed with detergent-lysates from RS11846 cells. After thorough washing, the beads were pelleted by centrifugation and associated proteins were subjected to SDS-PAGE/immunoblot assays with anti-CD40 antibody, demonstrating the presence of CD40 in samples containing the GST-CAP-1 protein but not the GST control protein (Fig. 1B).

3.4. Determination of the complete open reading frame of the CAP-1 protein reveals a new member of the TRAF family of putative signal transducing proteins

A human fetal brain cDNA library was screened and two additional overlapping clones having insert sizes of ~2.0 kbp and 0.1 kbp were obtained. The DNA sequence of the longer

of these extended the coding region in the 5'-direction by another 282 amino-acids but suggested that the complete ORF had still not been obtained. A 5'-RACE (rapid amplification of cDNA ends) procedure was then used to obtain an additional ~378 bp of DNA sequence. Taken together these cDNA cloning results demonstrated the presence of a continuous ORF predicted to encode a 543 amino-acid protein, having an estimated molecular mass of ~62 kDa (Fig. 2). The predicted translation initiation site conforms well to the Kozak consensus sequence, with 6 of 7 matches. Stop codons were found in all three reading-frames upstream of the longest ORF (nucleotide sequence submitted to Genbank).

A search of the CAP-1 nucleotide sequence against the available databases using the BLAST program revealed 26% and 30% overall amino-acid sequence homology with TRAF1 and TRAF2, two putative signal transducing proteins that have recently been shown to bind to the cytosolic domain of TNF-R2 [23]. The strongest homology was located in the C-terminal regions of these proteins, corresponding to the 'TRAF' domains of TRAF1 and TRAF2 (Fig. 2). The TRAF domain of CAP-1 is located between residues 384 to 540, and has 57% and 59% amino-acid identity to the analogous domains in TRAF1 and TRAF2, respectively.

Like TRAF2, the CAP-1 protein contains a RING finger domain near its NH_2-terminus (residues 53–91) having the consensus motif $C\text{-}X_2\text{-}C\text{-}X_{11-12}\text{-}C\text{-}X_{1-3}\text{-}H\text{-}X_2\text{-}C\text{-}X_2\text{-}C\text{-}X_{10-13}\text{-}C\text{-}X_2\text{-}C$. This sequence is believed to form a structure that binds two zinc atoms and is found in several regulatory proteins that can bind to either DNA or RNA, including the DNA repair genes RAD18 of *S. cerevisiae* and UVS-2 of *Neurospora crassa*, the human SS-A/Ro ribonucleoprotein, the Bmi-1, C-dbl, Pml, and RET proto-oncogenes, the recombinase RAG-1, and the human RING-1 protein (reviewed in [24]). The RING finger domains of CAP-1 and TRAF2 are 42% identical.

Following the RING finger motif are two zinc finger domains in CAP-1 that contain the consensus sequence $C/H\text{-}X_{2-4}\text{-}C/H\text{-}X_{2-15}\text{-}C/H\text{-}X_2\text{-}C/H$ which is found in several DNA-binding proteins including the *Xenopus* transcription factor TFIIIA [25] as well as some RING-finger proteins such as RAD18, and UVS-2 (reviewed in [24]). A third zinc-finger-like domain is found in CAP-1 (between residues 117–141), but the spacing between the first two cysteines is longer than usual in this case (6 instead of usual 2 to 4). The TRAF2 protein also contains four zinc finger motifs downstream of its RING finger domain [23]. Taken together, these data indicate that CAP-1 is a novel homolog of TRAF2 and a new member of the family of putative signal transducing proteins that contain TRAF-domains.

4. Discussion

Using a yeast two-hybrid approach, cDNAs encoding a novel protein CAP-1 that binds specifically to the cytosolic domain of CD40 were obtained. The region in the cytosolic domain of CD40 that is sufficient for CAP-1 binding constitutes a 45 amino-acid domain (residues 225–269) that is weakly homologous (22–41%) to domains found in the cytosolic portions of TNF-R1, Fas, and p75-NGF-R. This domain has been shown to be required for TNF-R1- and Fas-based cytotoxicity [15,16], suggesting that it plays an important role in some aspect of signal transduction mediated by these receptors. Conceivably, therefore, other TRAF-domain proteins could interact with these homologous domains in Fas, TNF-R1, and p75-NGF-R.

Previous analysis of the TRAF1 and TRAF2 proteins that bind to TNF-R2 failed to delineate the significance of the conserved C-terminal TRAF domain. Since the CAP-1 cDNA originally cloned during two-hybrid-based cDNA library screening encodes little more than the TRAF domain and given that this portion of CAP-1 (residues 384–540) is sufficient for interactions with the cytoplasmic domain of CD40 both in vitro and in two-hybrid assays, this finding suggests that one function of the TRAF domain is to mediate interactions with TNF-R family receptors. In addition, we showed that this same region in CAP-1 is sufficient to mediate dimerization of CAP-1 fusion proteins, suggesting yet another role for the TRAF domain. Previously it was shown that TRAF1 and TRAF2 can form homotypic and heterotypic dimers [23]. It is presently unknown whether CAP-1 can dimerize with TRAF1 or TRAF2. By analogy, however, it seems likely that CAP-1 will be able to participate in heterodimer formation with at least some other known or unknown members of the TRAF family, since the TRAF-domain in CAP-1 appears to be sufficient for dimerization with itself. This possibility for various combinations of homo- and heterotypic dimers could then create opportunities for specificity in signal transduction response which might be controlled both at the level of gene expression in terms of which members of the TRAF-domain gene family that are expressed in any particular cell-type and at the level of protein-protein interactions between TRAF-domain-containing proteins and specific members of the TNF-R family. With regards to the former, preliminary results suggest that CAP-1 is widely expressed (our unpublished data), like TRAF2, but unlike TRAF-1 which appears to be expressed in only spleen, lung, and testes [23]. However, specificity in the involvement of CAP-1 in signal transduction presumably can be achieved through its ability to interact with CD40 but not several other members of the TNF-R family, including TNF-R1, TNF-R2, and Fas.

CAP-1 and TRAF-2 both contain RING finger domains near their N-termini. This cysteine-rich structure represents a unique zinc-binding motif that has been found in over 40 proteins to date (reviewed in [24]). Though RING-fingers may have other roles, in at least some circumstances they have been shown to bind to DNA [26–28]. Moreover, several viral transcriptional regulators including the Herpes Simplex virus immediate-early genes IEHBV, IEEHV, and IE110 contain RING fingers and deletion of this domain in IE110 has been reported to abolish its function as a *trans*-activator [29,30]. These findings raise the possibility that CAP-1 and TRAF2 may participate directly in signal transduction from membrane to nucleus by analogy to the Stat proteins, which associate with the cytoplasmic domains of many cytokine receptors, dissociate from the receptor at the membrane upon ligand-induced phosphorylation, and translocate into the nucleus where they bind to specific DNA sequences and participate in the regulation of gene expression (reviewed in [31]). Interestingly, CAP-1 also contains three and TRAF2 has two zinc-finger domains similar to those first found in the *Xenopus* transcription factor TFIIIA [25], thus further suggesting a potential role for these proteins in transcriptional regulation. However, at present it is unknown whether RING-finger domains constitute sequence-specific DNA-binding elements, and other potential functions for proteins that contain this structure are clearly possible, including

participation in protein-protein and protein-lipid interactions [24]. Though the precise role that CAP-1 plays in CD40-mediated signal transduction responses remains to be determined, the identification of CAP-1 and the demonstration of its homology with other TRAF-domain-containing proteins nevertheless represent significant advances in our understanding of the mechanisms by which CD40 and some other members of the TNF-R family exert their potent biological effects in cells.

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