Herpesvirus Entry Mediator, a Member of the Tumor Necrosis Factor Receptor (TNFR) Family, Interacts with Members of the TNFR-associated Factor Family and Activates the Transcription Factors NF-κB and AP-1*

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The mammalian tumor necrosis factor receptor (TNFR) family consists of 10 cell-surface proteins that regulate development and homeostasis of the immune system. Based on an expressed sequence tag, we have cloned a cDNA encoding a novel member of the human TNFR family. A closely related protein, designated HVEM (for herpesvirus entry mediator), was identified independently by another group as a mediator of herpesvirus entry into mammalian cells (Montgomery, R., Warner, M., Lum, B., and Spear, P. (1996) Cell 87, 427–436). HVEM differed from our clone by two amino acid residues, suggesting that the two proteins represent polymorphism of a single HVEM gene. We detected HVEM mRNA expression in several human fetal and adult tissues, although the predominant sites of expression were lymphocyte-rich tissues such as adult spleen and peripheral blood leukocytes. The cytoplasmic region of HVEM bound to several members of the TNFR-associated factor (TRAF) family, namely TRAF1, TRAF2, TRAF3, and TRAF5, but not to TRAF6. Transient transfection of HVEM into human 293 cells caused marked activation of nuclear factor-κB (NF-κB), a transcriptional regulator of multiple immunomodulatory and inflammatory genes. HVEM transfection induced also marked activation of Jun N-terminal kinase, and of the Jun-containing transcription factor AP-1, a regulator of cellular stress-response genes. These results suggest that HVEM is linked via TRAFs to signal transduction pathways that activate the immune response.

Members of the TNFR1 family play a key role in regulating the immune response to infection. For example, TNFR1, TNFR2, and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor NF-κB (2). Some TNFR family members regulate also the AP-1 transcription factor, whose target genes are less well defined (3). NF-κB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions (4). In its latent form, NF-κB is complexed with members of the IκB inhibitor family; upon inactivation of IκB in response to certain stimuli, released NF-κB translocates to the nucleus, where it binds to specific DNA sequences and activates gene transcription (4). AP-1 represents a family of dimeric complexes composed of members of the Jun and Fos protein families (3). AP-1 activation is mediated in part through phosphorylation of Jun proteins by Jun N-terminal kinases (JNKs), also known as stress-activated protein kinases (SAPKs) (3, 5).

Transcriptional regulation by TNFR family members is mediated by a family of signal transducers known as TNFR-associated factors (TRAFs) (6). For example, TRAF2 associates directly with TNFR2, CD40, and CD30 and plays a key role in NF-κB activation by these receptors (6–8). TRAF2 also mediates TNFR1 activation of NF-κB and of JNK, although it associates with TNFR1 indirectly, via the TRADD adaptor protein; TRADD signals apoptosis activation by TNFR1 as well (9, 10). TRAF1 associates with TNFR2 indirectly, by interaction with TRAF2 (6), while TRAF3/CD40bp/CRAF1 associates directly with and mediates signaling by CD40 and the lymphotixin β receptor (LTbR) (7, 11–13), as does TRAF5 (14, 15). TRAF6 binds to CD40 and contributes to CD40 signaling (16), as well as to signaling by interleukin-1 (17).

By using an expressed sequence tag (EST) approach, we have identified a member of the TNFR family that proved to be closely related to the recently isolated HVEM (18). In the present study, we provide evidence suggesting that HVEM interacts with several members of the TRAF family and modulates activation of NF-κB, JNK, and AP-1.

EXPERIMENTAL PROCEDURES

Analysis of HVEM mRNA Expression—Human fetal and adult tissues poly(A) RNA blots (CLONTECH) were analyzed by Northern hybridization using a 32P-labeled cDNA probe containing the entire HVEM coding region. In addition, poly(A) RNA was prepared from purified human peripheral blood T cells (19) and from Jurkat T cells (ATCC) using a FastTrack kit (Invitrogen), and 50 ng of RNA were analyzed for HVEM expression by reverse transcriptase-polymerase chain reaction (PCR) using oligonucleotide primers that amplify a 240-base pair sequence in the putative extracellular region of HVEM.

Interaction of HVEM with TRAFs—The cytoplasmic region of HVEM was cloned into the pGEX-2TK vector (Pharmacia Biotech Inc.), expressed in Escherichia coli as a fusion protein with glutathione S-transferase (GST), and subsequently purified by glutathione-agarose affinity chromatography (20). HEK293 cells (ATCC) were transiently transfected by the calcium phosphate precipitation method with pRK5-based expression vectors encoding Flag epitope-tagged TRAFs 1, 2, 3, 5, or 6 (5 μg/10-cm dish). Total cell lysates were prepared 36 h later (7). Aliquots from each lysate were incubated for 2 h at 4 °C with ~1 μg of GST-HVEM fusion protein. Bound complexes were precipitated with

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The abbreviations used are: TNFR, tumor necrosis factor receptor; EMSA, electrophoretic mobility shift assay; EST, expressed sequence tag; JNK, Jun N-terminal kinase; GST, glutathione S-transferase;
glutathione-agarose beads, resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblot with anti-Flag monoclonal antibody (Eastman Kodak Co.) using the enhanced chemiluminescence Western blotting detection system (Amersham). The interaction of GST-HVEM with endogenous TRAFs in nontransfected HEK293 cells was determined using a similar approach, except that the TRAFs were detected by sequential immunoblot with polyclonal anti-human TRAF1 or TRAF2 antibodies (21), 4E7 anti-human TRAF3 monoclonal antibody (Bios), or polyclonal anti-murine TRAF5 antibody (Zymed), which cross-reacts with human TRAF5.  

Electrophoretic Mobility Shift Assay (EMSA)—The activation of NF-κB and AP-1 was analyzed by EMSA using oligonucleotide probes specific for each transcription factor. Nuclear extracts were prepared 24 h after transient transfection (22) of HEK293 cells by HVEM or control expression plasmids (10 μg/10-cm dish). Aliquots from each extract (1 μg of total protein) were reacted (22) with a 32P-labeled oligonucleotide probe. For NF-κB, we used as a control a radioprobe based on a mutated NF-κB target sequence (plus strand sequence 5′-AGTTGAGGGAGTTCCAGGGCC-3′) (23), or a radioprobe based on a wild type NF-κB target sequence (plus strand sequence 5′-AGTTGAGGGAGTTCCAGGGCC-3′) (24). For AP-1 we used a control radioprobe based on a mutated AP-1 target sequence (plus strand sequence 5′-GGCTTGATGACTGGAGGGGAA-3′) (24), or a radioprobe based on a wild type AP-1 target sequence (plus strand sequence 5′-GGCTTGATGACTGGAGGGGAA-3′) (24). The reactions were subjected to PAGE and visualized by phosphorimager analysis (22).

JNK Activation Assay—JNK activation was determined with a SAPK/JNK assay kit (New England Biolabs). Cell lysates were prepared 24 h after transient transfection of HEK293 cells by HVEM or control expression plasmids. JNK was precipitated with a GST-c-Jun fusion protein bound to glutathione-Sepharose beads. After washing, the kinase reaction was allowed to proceed in the presence of ATP, and was resolved by SDS-PAGE. Phospho-c-Jun was detected by immunoblot with antibody specific for c-Jun phosphorylated on Ser-63, a site important for transcriptional activity (5), using chemiluminescence.

RESULTS AND DISCUSSION

TNFR family members share sequence homology primarily in their extracellular region, which contains three to six characteristic cysteine-rich pseudorepeats (1, 25). To identify new members of the TNFR family, we searched the DNA data bases for ESTs that exhibit homology to individual TNFR family members. We identified a human retinal EST (GenBank locus AA021617), which upon translation showed homology to the cysteine-rich regions of several TNFR family proteins. To isolate the full-length cDNA, we screened a human retinal cdna bacteriophage library (CLONTECH) by hybridization to a 60-base-pair oligonucleotide probe based on a region of consensus between AA021617 and several related ESTs. We identified five independent positive clones containing cDNA inserts of 1.8–1.9 kb. The three of the inserts were subcloned into the pBluescript plasmid, and sequenced on both strands. The cDNA sequences were identical (with the exception of an intron found in one of the clones), and encoded a putative 283-amino acid transmembrane protein that showed significant extracellular sequence homology to the TNFR family (data not shown). Subsequently, a protein designated HVEM was reported independently (18), and turned out to be closely related to the protein we had identified. HVEM was isolated from a human HeLa cell cDNA library as a mediator of herpesvirus entry into mammalian cells; the cloning strategy was based on a functional screen of transfected cDNA clones for ability to confer herpesvirus sensitivity upon resistant cells (18). The protein we identified and HVEM (18) differ, respectively, in two extracellular amino acid residues: codon 108 encodes a serine or a threonine, and codon 140 encodes an alanine or an arginine. These differences may be due to polymorphism in the HVEM gene; hence, we refer to both proteins here as HVEM.

We investigated expression of the HVEM mRNA in human tissues by Northern blot hybridization (Fig. 1, A–C). We detected a major transcript of about 1.8 kb, which is similar to the size of our cDNA clones, in multiple fetal and adult tissues; this transcript was most highly expressed in adult spleen and peripheral blood leukocytes. A second transcript of about 3.8 kb, as well as some larger transcripts also were detected. In addition, we analyzed the expression of HVEM mRNA in peripheral T cells purified from human blood and in the human Jurkat T cell line by reverse transcriptase-PCR (Fig. 1D). Both primary T cells and the T cell line expressed HVEM mRNA. The data of Fig. 1B are consistent with the previous observation that HVEM mRNA is expressed in several non-lymphoid tissues (18). However, our results demonstrate that in fact the predominant sites of HVEM mRNA expression are lymphocyte-rich tissues, i.e. spleen and peripheral blood leukocytes (Fig. 1C), and show further that HVEM mRNA is expressed in purified T cells (Fig. 1D) and in several fetal tissues (Fig. 1A).

To investigate whether HVEM interacts with TRAFs, we generated a GST fusion protein based on the cytoplasmic region of HVEM and tested its ability to co-precipitate five of the six known TRAFs upon their overexpression in HEK293 cells (Fig. 2A and B). We observed strong association of HVEM with TRAF2; weaker association with TRAF5, TRAF3, and TRAF1; and no association with TRAF6. Consistent with these results, HVEM bound also to endogenous TRAFs in HEK293 cells, with a similar rank order (Fig. 2C). LTβR recognized the same set of endogenous TRAFs in HEK293 cells as did HVEM, while TNFR2 interacted mainly with TRAF2 and TRAF1 (Fig. 2C). These results are intriguing, because the 58-amino acid cytoplasmic region of HVEM is related to the cytoplasmic domain of LTβR (11 identities), but shows no homology to the cytoplasmic region of TNFR2. CD40, another TNFR family member that shows significant cytoplasmic region homology to HVEM (12 identities), also interacts with TRAF2, TRAF3, and TRAF5 (7, 11, 12), but unlike HVEM, it recognizes TRAF6 as well (16).

To explore possible HVEM-regulated signaling pathways, we investigated whether transfection of HEK293 cells by HVEM affects NF-κB activity (Fig. 3). Cells transfected by a pRK5-based HVEM expression plasmid showed significant NF-κB activation relative to cells transfected by pRK5 alone (Fig. 3A). For comparison, we tested two TNFR family members that have been shown previously to activate NF-κB, namely
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Fig. 2. HVEM associates with specific members of the TRAF family. A, interaction of HVEM with overexpressed TRAFs was analyzed by incubation of purified GST-fusion protein containing the HVEM cytoplasmic domain with lysates from HEK293 cells transiently transfected with expression vectors encoding Flag-epitope-tagged TRAFs 1, 2, 3, 5, or 6. Bound complexes were precipitated with glutathione-agarose, resolved by SDS-PAGE, and detected by immunoblot analysis with anti-Flag monoclonal antibody. B, the total amount of transfected TRAF protein in each reaction was assessed by direct immunoprecipitation of equally sized aliquots of cell lysates with 5 μg of the anti-Flag antibody and 30 μl of protein G-agarose, and immunoblot analysis as above. C, interaction of HVEM with endogenous TRAFs in HEK293 cells. GST fusions based on the cytoplasmic region of HVEM, LTβR, or TNFR2 were used in co-precipitation reactions as above, and associated TRAFs were identified by sequential immunoblot with antibodies specific to the indicated TRAFs.

Fig. 3. HVEM transfection causes activation of NF-κB. A, HEK293 cells were transiently transfected with pRK5, or with pRK5-based expression vectors encoding HVEM, TNFR1, or Apo-3, and analyzed 24 h later by NF-κB EMSA. The extracts were reacted with a 32P-labeled oligonucleotide based on a mutated NF-κB target sequence (Control radioprobe), or with a 32P-labeled oligonucleotide based on a wild type NF-κB target sequence added alone (NF-κB radioprobe) or together with a 50-fold excess of unlabeled oligonucleotide of the same sequence (+ cold oligo). The NF-κB-specific band is indicated by an arrow. B, nuclear extracts were incubated with the NF-κB-specific radioprobe in the presence of rabbit preimmune serum or of rabbit anti-p65/RelA IgG (Santa Cruz Biotechnology), and analyzed by EMSA. The positions of the nonshifted and the antibody-shifted NF-κB probe are indicated by arrows.

Fig. 4. HVEM transfection causes activation of JNK and AP-1. HEK293 cells were transiently transfected with pRK5, or with pRK5-based expression vectors encoding HVEM or TNFR1, and analyzed 24 h later for JNK or AP-1 activity. A, JNK activity was assayed by co-precipitating cellular JNK with GST-c-Jun, adding ATP, and detecting phospho-c-Jun by immunoblot with an antibody specific for c-Jun phosphorylated on Ser-63, a site important for transcriptional activity. B, AP-1 activity was determined by EMSA, using a 32P-labeled oligonucleotide based on a mutated AP-1 target sequence (Control radioprobe), or a 32P-labeled oligonucleotide based on a wild type AP-1 target sequence added alone (AP-1 radioprobe) or together with a 50-fold excess of unlabeled oligonucleotide of the same sequence (+ cold oligo). The AP-1-specific band is indicated by an arrow. C, nuclear extracts were incubated with the AP-1-specific radioprobe together with rabbit preimmune serum or with rabbit anti-Jun D IgG (Santa Cruz Biotechnology), and analyzed by EMSA. The positions of the nonshifted and the antibody-shifted AP-1 probe are indicated by arrows.

TNFR1 and Apo-3/DR-3/WSL-1 (2, 26–28); the level of NF-κB activation by these receptors was similar to the level of activation by HVEM (Fig. 3A). A specific antibody to the p65/RelA subunit of NF-κB, but not preimmune serum, inhibited the mobility of the NF-κB probe in the case of each receptor (Fig. 3B). Hence, the NF-κB complexes activated by HVEM, TNFR1, and Apo-3 in HEK293 cells appear to contain the p65/RelA protein (Fig. 3B). These results indicate that HVEM is linked to an NF-κB activation pathway. It is possible that interaction of HVEM with TRAF2 and/or TRAF5 (Fig. 2) mediates the NF-κB activation, since these TRAFs are key to NF-κB activation by other related TNFR family members (see above).

Next, we investigated whether transfection of HEK293 cells by HVEM activates JNK, as well as the transcription factor AP-1, which is activated through JNK-mediated phosphorylation of Jun proteins (Fig. 4). HVEM-transfected cells showed marked JNK activation as compared with cells transfected by pRK5 alone (Fig. 4A); the level of activation was comparable to the level induced by TNFR1 transfection. Consistent with JNK activation, HVEM-transfected cells showed AP-1 activation as well (Fig. 4B). Anti-Jun D antibody, but not preimmune serum, inhibited the migration of the AP-1-specific probe (Fig. 4C), suggesting that Jun D participates in the AP-1 complexes activated by HVEM in HEK293 cells. These results indicate that HVEM is coupled to the JNK/AP-1 signaling pathway. The interaction of HVEM with TRAF2 (Fig. 2) may contribute to JNK/AP-1 activation by HVEM, since TRAF2 plays a key role in mediating JNK activation by TNFR1 (10). However, it remains to be established which TRAFs signal NF-κB and JNK/AP-1 activation by HVEM.

CONCLUSION

Using an EST-based approach, we have identified a novel member of the human TNFR family. Montgomery et al. (18) isolated a closely related protein, HVEM, as an entry receptor for herpesvirus. There are only two amino acid differences between the two receptors, suggesting that these proteins may be encoded by a single, polymorphic HVEM gene. Our results provide an important step toward understanding the physiological function of HVEM. HVEM associates with members of the TRAF family and activates JNK/SAPK, as well as the transcription factors NF-κB and AP-1, which control expression of multiple immune, inflammatory, and acute phase action genes in response to infection or cellular stress (3–5). The induction of NF-κB and AP-1 by HVEM, together with the relatively abundant expression of HVEM mRNA in T cells and in lymphocyte-rich tissues, suggests that HVEM is involved in regulating lymphocyte activation.
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