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LMP1 signal transduction differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2

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The Epstein–Barr virus latent membrane protein 1 (LMP1) binds tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) and the TNFR-associated death domain protein (TRADD). Moreover, it induces NF-κB and the c-Jun N-terminal kinase 1 (JNK1) pathway. Thus, LMP1 appears to mimick the molecular functions of TNFR1. However, TNFR1 elicits a wide range of cellular responses including apoptosis, whereas LMP1 constitutes a transforming protein. Here we mapped the JNK1 activator region (JAR) of the LMP1 molecule. JAR overlaps with the TRADD-binding domain of LMP1. In contrast to TNFR1, LMP1 recruits TRADD via the TRADD N-terminus but not the TRADD death domain. Consequently, the molecular function of TRADD in LMP1 signaling differs from its role in TNFR1 signal transduction. Whereas NF-κB activation by LMP1 was blocked by a dominant-negative TRADD mutant, LMP1 induces JNK1 independently of the TRADD death domain and TRAF2, which binds to TRADD. Further downstream, JNK1 activation by TNFR1 involves Cdc42, whereas LMP1 signaling to JNK1 is independent of p21 Rho-like GTPases. Although both LMP1 and TNFR1 interact with TRADD and TRAF2, the different topologies of the signaling complexes correlate with substantial differences between LMP1 and TNFR1 signal transduction to JNK1.

Keywords: c-Jun N-terminal kinase 1/latent membrane protein 1/nuclear factor-κB/signal transduction/TNF receptor 1

Introduction

Latent membrane protein 1 (LMP1) is the major oncogene of the human DNA tumor virus EBV (Epstein–Barr virus). Persistent EBV infection can result in the development of malignant diseases such as Hodgkin's lymphoma, Burkitt's lymphoma, nasopharyngeal carcinoma or post-transplant lymphoproliferative diseases (reviewed in Klein, 1994; Kieff, 1996; Niedobitek *et al.*, 1997). EBV efficiently infects and immortalizes resting B cells *in vitro* (reviewed in Farrell, 1995; Kieff, 1996). LMP1 expression is absolutely required for the immortalization process (Kaye *et al.*, 1993; Kilger *et al.*, 1998). Moreover, LMP1 constitutes a genuine viral oncogene since it transforms rodent fibroblasts *in vitro* (Wang *et al.*, 1985; Baichwal and Sugden, 1988; Moorthy and Thorley-Lawson, 1993). Transgenic mice carrying an Ig heavy chain promoter/enhancer-driven LMP1 gene develop lymphomas with high incidence *in vivo* (Kulwichit *et al.*, 1998), demonstrating that LMP1 alone is a transforming gene even in situations lacking an EBV background.

LMP1 most likely immortalizes and transforms cells by simultaneously controlling cellular signaling pathways that block apoptosis or mediate proliferative, growth factor-like effects. LMP1 induces the expression of the anti-apoptotic genes *bcl-2* (Henderson *et al.*, 1991) and *A20*, the latter via induction of the transcription factor NF-κB (Laherty *et al.*, 1992). The transforming potential of LMP1, however, does not appear to depend on NF-κB activation or induction of *bcl-2*, since LMP1 transforms BALB/3T3 cells without inducing NF-κB or *bcl-2* (Baichwal and Sugden, 1988; Martin *et al.*, 1993; Mitchell and Sugden, 1995). We and others have shown that LMP1 induces the mitogenic transcription factor AP1, a dimer of Jun–Jun or Jun–Fos family proto-oncoproteins. LMP1 induces AP1 activity specifically via the c-Jun N-terminal kinase 1 (JNK1) pathway (Kieser *et al.*, 1997; Eliopoulos and Young, 1998). LMP1 activity causes the JNK1-mediated phosphorylation of c-Jun and a concomitant up-regulation of c-Jun transcriptional transactivation (Kieser *et al.*, 1997). It is believed that JNK1 is involved in the apoptotic response of cells to environmental stress (reviewed in Kyriakis and Avruch, 1996). More recently, it became evident that JNK1 is also part of non-cytotoxic signaling events triggered by CD40 (Berberich *et al.*, 1996), tumor necrosis factor receptor 1 (TNFR1, 55 kDa) (Liu *et al.*, 1996; Natoli *et al.*, 1997) or epidermal growth factor (Bost *et al.*, 1997). We could show that activation of endogenous JNK1 by LMP1 correlates with a strong induction of proliferation but not apoptosis in mini-EBV-immortalized primary human B cells (Kieser *et al.*, 1997; Kilger *et al.*, 1998).

LMP1 is an integral membrane protein consisting of 386 amino acids. As shown in Figure 1, six transmembrane-spanning domains (162 amino acids) connect a short N-terminal stretch (24 amino acids) with a long C-terminal domain (200 amino acids), both of which are located in the cytoplasm (Liebowitz *et al.*, 1986; Kieff, 1996). LMP1 acts as a constitutively active receptor-like molecule independently of the binding of a ligand (Gires *et al.*, 1997). The six transmembrane domains mediate oligomerization of LMP1 molecules in the plasma membrane, a prerequisite for LMP1 function (Floettmann and Rowe, 1997; Gires *et al.*, 1997). So far, two domains in the C-terminus of LMP1 have been shown to initiate signaling processes, the C-terminal activator regions 1 (CTAR1, amino acids 194–231) and 2 (CTAR2, amino acids 332–386) (Huen *et al.*, 1995; Mitchell and Sugden, 1995).

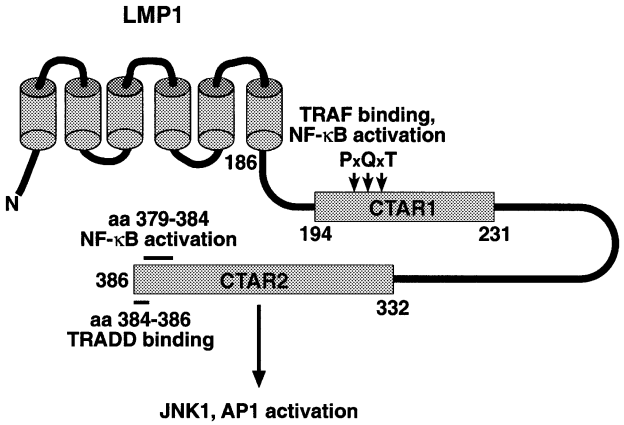

Fig. 1. The functional domains of the LMP1 molecule. Numbers indicate amino acid (aa) positions. Amino acids 1–186 comprise the short N-terminus and the six transmembrane domains. The CTAR1 and CTAR2 domains are involved in the induction of signaling processes. CTAR1 contains a PxQxT motif responsible for TRAF binding and CTAR1-dependent NF-κB activation. Amino acids 384–386 of CTAR2 are essential for the binding of TRADD, and amino acids 379–384 for CTAR2-derived NF-κB induction. JNK1/AP1 signaling is triggered solely at CTAR2. For a more detailed description, see text.

Both domains are involved in the induction of NF-κB, CTAR2 being the principal NF-κB activator site. Approximately 70% of the total NF-κB induction capacity of LMP1 is delivered by the CTAR2 domain, and the remaining 30% is derived from CTAR1 (Huen *et al.*, 1995; Mitchell and Sugden, 1995). Amino acids 204–208 in CTAR1 comprise a consensus motif for binding TNFR-associated factors (TRAFs), the PxQxT motif (Devergne *et al.*, 1996). TRAFs 1, 2, 3 and 5 were shown to bind to CTAR1 (Mosialos *et al.*, 1995; Devergne *et al.*, 1996; Brodeur *et al.*, 1997; Sandberg *et al.*, 1997). In contrast, a direct interaction of TRAFs with CTAR2 could not be demonstrated. Nevertheless, NF-κB signaling by both domains is impaired by dominant-negative mutants of TRAF2 and of the TRAF2-associated MAPKK kinase NIK (NF-κB-inducing kinase) (Devergne *et al.*, 1996; Kaye *et al.*, 1996; Sylla *et al.*, 1998). This observation suggests that NF-κB signaling of both domains converges at the TRAF2–NIK module. Recently, it has been shown that LMP1 recruits the TNFR-associated death domain protein (TRADD) via sequences in its very C-terminus (Izumi and Kieff, 1997). TRADD directly interacts with TNFR1 and is involved in the induction of NF-κB and apoptosis by TNFR1 (Hsu *et al.*, 1995). Moreover, TRADD binds TRAF2 (Hsu *et al.*, 1996b). Thus, TRADD has been suggested to act as a bridging protein between CTAR2 and TRAF2, mediating CTAR2 signaling to NF-κB (Izumi and Kieff, 1997; Sylla *et al.*, 1998), but a role for TRADD in LMP1 signaling has not been proven formally. In contrast to NF-κB, JNK1 induction by LMP1 is triggered solely by the CTAR2 domain (Kieser *et al.*, 1997; Eliopoulos and Young, 1998). The signaling mechanisms linking CTAR2 with the JNK1 module are completely unknown.

As discussed above, both LMP1 and TNFR1 interact with the adaptor molecules TRADD and TRAF2 and both induce NF-κB, JNK1 and AP1, suggesting that LMP1 mimicks an activated TNFR1 (reviewed in Yuan, 1997; Farrell, 1998). However, profound differences exist between the effects of both receptor molecules within the cell. Whereas TNFR1 elicits a broad range of cellular responses including apoptosis (Yuan, 1997), LMP1 constitutes a transforming protein. Moreover, TRADD binds to TNFR1 by interaction of the death domains of both molecules (Hsu *et al.*, 1995), but such an interaction is not possible between LMP1 and TRADD since LMP1 does not contain a death domain. The death domain is a protein–protein interaction domain of ~80 amino acids conserved between several apoptosis-inducing receptors such as TNFR1 (Tartaglia *et al.*, 1993) or death receptor 3 (Chinnaiyan *et al.*, 1996), and death-signaling molecules such as TRADD (Hsu *et al.*, 1995), Fas-associating death domain protein (FADD) (Chinnaiyan *et al.*, 1995) or the receptor-interacting protein kinase (RIP) (Stanger *et al.*, 1995; Hsu *et al.*, 1996a). So far, the TRADD domains necessary for LMP1 binding have not been defined.

Here, we identify the JNK1 activator region of the LMP1 molecule as a subdomain of six amino acids in CTAR2, a region which overlaps with the TRADD interaction domain of LMP1. This led us to investigate a potential role for TRADD and TRAF2 in LMP1 signaling to JNK1 and to NF-κB. We provide experimental evidence that the molecular architecture of the LMP1–TRADD complex differs from that of the TNFR1–TRADD complex. This observation is reflected by substantial differences in JNK1 signaling between LMP1 and TNFR1 with respect to the role of TRADD/TRAF2 and, further downstream, to the role of p21 Rho-like GTPases. In contrast, NF-κB signaling of LMP1 and TNFR1 appears to function through similar mechanisms. Our results might help to understand why LMP1 and TNFR1 elicit different cellular responses although they act via similar adaptor molecules.

Results

Mapping of the JNK1 activator region of the LMP1 molecule

In order to investigate the mechanism by which LMP1 induces JNK1 activity, it was necessary to identify the precise JNK1 activator domain of the LMP1 molecule. By deletion analysis, we and others have shown that the CTAR2 domain is essential for JNK1 activation by LMP1 (Kieser *et al.*, 1997; Eliopoulos and Young, 1998). So far, the only known CTAR2-binding molecule is TRADD, and the last three C-terminal amino acids of LMP1 (Y384, Y385 and D386) are essential for TRADD binding (Izumi and Kieff, 1997). To determine which part of the CTAR2 domain is involved in JNK1 activation, LMP1 mutants were tested in transient transfection assays. For that purpose, we transfected LMP1 expression vectors together with a hemagglutinin (HA)-tagged JNK1 (HA-JNK1) into 293 cells, subsequently immunoprecipitated HA-JNK1 from cleared cell lysates and assayed for HA-JNK1 activity in *in vitro* kinase assays using purified GST-tagged c-Jun (GST–c-Jun) as a substrate.

As shown in Figure 2A, the functional knockout of CTAR1 had no effect on JNK1 induction by LMP1. The mutants LMP1Δ212–231 lacking amino acids 212–231 (Kieser *et al.*, 1997; Sandberg *et al.*, 1997) and LMP1(PQT→AAA) carrying a mutated PxQxT motif (PxQxT mutated to AxAxA) (Devergne *et al.*, 1996;

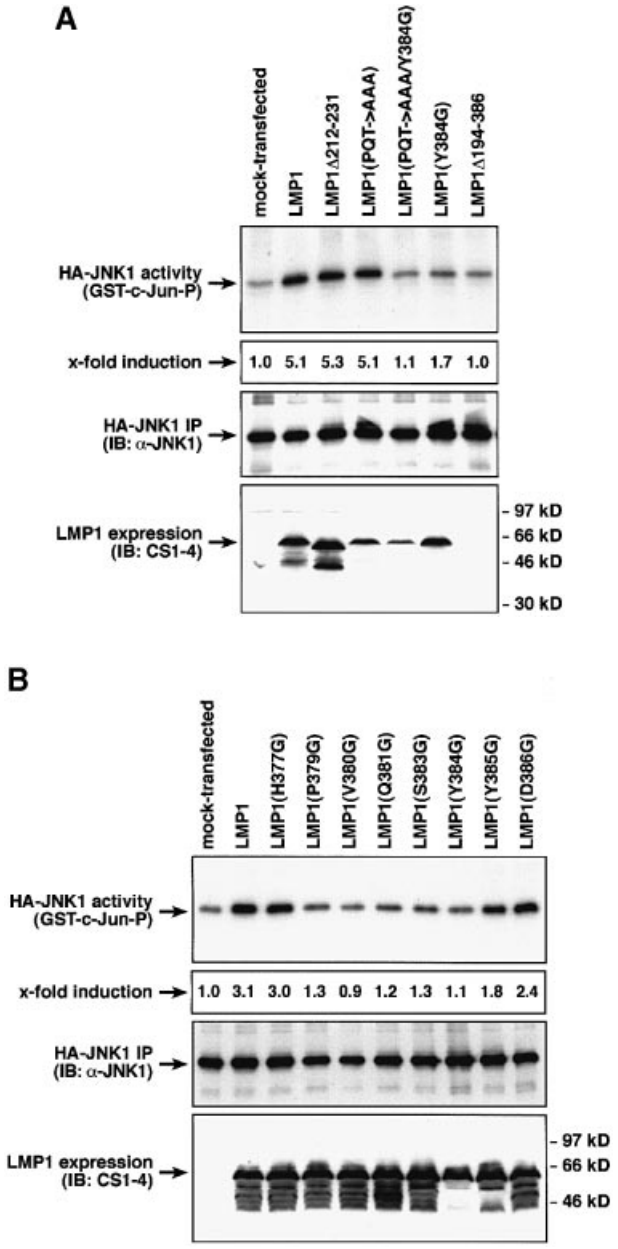

Fig. 2. Mapping of the JNK1 activator region (JAR) of LMP1. (**A**) Tyr384 but not the CTAR1 domain is essential for JNK1 activation by LMP1. One microgram of the HA-JNK1 expression vector pSRα-HA-JNK1 was co-transfected with 2 μg of pSV-LMP1, pSV-LMP1Δ212–231, pSV-LMP1(PQT→AAA), pSV-LMP1(PQT→AAA/Y384G), pSV-LMP1(Y384G) or pSV-LMP1Δ194–386, or 2 μg of carrier DNA for a mock-transfected control, as indicated. At 24 h post-transfection, HA-JNK1 immunocomplex kinase assays were performed. Top panel: autoradiograph of GST–c-Jun *in vitro* phosphorylated by immunoprecipitated HA-JNK1. Second panel: x-fold induction of HA-JNK1 kinase activities as quantitated by phosphoimager analysis. Third panel: immunoblotting analysis (IB) of the immunoprecipitated (IP) HA-JNK1 protein using the rabbit anti-JNK1 C-17 antibody. Bottom panel: immunoblotting analysis of LMP1 expression. Apparent molecular weights are given in kiloDaltons. (**B**) Amino acids 379–384 comprise the core JNK1 activator region. One microgram of pSRα-HA-JNK1 was co-transfected with 2 μg of pSG5-LMP1, the indicated LMP1 amino acid exchange mutants based upon pSG5-LMP1, or pSG5 for a mock-transfected control.

Eliopoulos *et al.*, 1997) do not bind TRAFs any more, but both CTAR1 mutants still induce JNK1 to wild-type LMP1 levels. In contrast, mutation of Y384 to glycine either in wild-type LMP1, LMP1(Y384G) or in the LMP1(PQT→AAA) mutant, LMP1(PQT→AAA/Y384G), completely abolished JNK1 induction. Thus, Tyr384 appears to be essential for JNK1 induction by LMP1. All mutants shown in Figure 2A are based on the pSV-LMP1 expression vector (Kieser *et al.*, 1997) expressing comparable levels of LMP1 proteins. The inactive LMP1Δ194–386 mutant lacking the complete C-terminal domain cannot be detected on immunoblots by the anti-LMP1 antibody CS1-4 since all CS1-4 epitopes are located within the LMP1 C-terminus (Rowe *et al.*, 1987; Floettmann and Rowe, 1997).

To map the complete subdomain responsible for JNK1 activation, we tested a series of LMP1 point mutants for their ability to induce JNK1. Single amino acids between positions 377 and 386 of LMP1 were changed into glycines (Floettmann and Rowe, 1997). The remaining positions 378 and 382 are glycine or leucine residues in wild-type LMP1, respectively. As shown in Figure 2B, mutation H377G had no effect on JNK1 induction by LMP1 in transient transfection assays. In contrast, mutations P379G, V380G, Q381G, S383G and Y384G led to a complete loss of LMP1's ability to induce JNK1. Notably, the two C-terminal mutations Y385G and D386G only caused a partial loss of LMP1's capacity to activate JNK1 (62 and 33% loss of induction, respectively), indicating that these two amino acids are not part of the core JNK1 activator region but rather disturb its function. Wild-type LMP1 and all LMP1 mutants used in this experiment were expressed from the pSG5 expression vector resulting in equal LMP1 expression levels (Figure 2B). In summary, we have identified a subdomain of six amino acids from positions 379 to 384 as the core JNK1 activator region (JAR) of the LMP1 molecule. Interestingly, JAR is identical to the NF-κB activator region of CTAR2 as mapped previously (Floettmann and Rowe, 1997). Moreover, JAR overlaps with the TRADD-binding domain of LMP1 (Izumi and Kieff, 1997).

The TRADD N-terminus binds to LMP1

To investigate further a potential role for TRADD in LMP1 signaling, we studied the interaction between TRADD and LMP1 in more detail. Mutational analysis revealed that the death domain-containing C-terminus of TRADD (amino acids 195–312) is necessary and sufficient for self-association and binding to the TNFR1 death domain as well as for induction of apoptosis and NF-κB (Hsu *et al.*, 1995; Park and Baichwal, 1996). As discussed above, LMP1 lacks a death domain and binds TRADD solely via a short sequence in its C-terminus. This led us to examine whether TRADD can interact with LMP1 independently of its death domain.

For this purpose, we used a fusion protein of GST and the C-terminal 205 amino acids (positions 181–386) of LMP1 (GST–LMP1) (Sandberg *et al.*, 1997), immobilized to glutathione–Sepharose beads, to precipitate transiently expressed TRADD or TRADD mutants from 293 cell lysates (Figure 3). As a negative control, GST-coupled beads were used in parallel pull-down experiments. Expression vectors coding for wild-type TRADD or TRADD mutants containing an N-terminal Myc epitope tag (Hsu *et al.*, 1995) were transiently transfected into 293 cells. The TRADD mutants were TRADD(1–194),

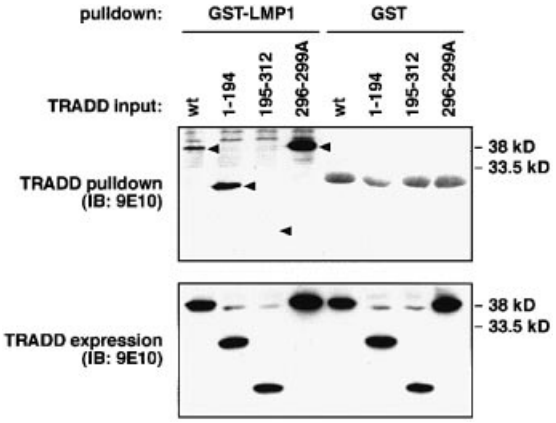

Fig. 3. The TRADD N-terminus binds to LMP1. 293 cells were transiently transfected with 125 ng of pRK-myc-TRADD, pRK-myc-TRADD(1–194) coding for the TRADD N-terminus, pRK-myc-TRADD(195–312) coding for the TRADD death domain, or pRK-myc-TRADD(296–299A), as indicated. pcDNA3-p35 (0.5 μg) was co-transfected in each well to protect cells from TRADD-induced apopotosis. At 24 h post-transfection, cells were lysed in TBST. The lower panel shows the expression of the Myc epitope-tagged TRADD proteins as detected in immunoblots of total cell lysates using the 9E10 antibody. TRADD proteins were precipitated from cell lysates with either GST–LMP1 (left column) or GST as a negative control (right column), both immobilized to glutathione–Sepharose beads. Upper panel: immunoblotting analysis of co-precipitated TRADD proteins using the 9E10 antibody. The arrowheads in the left column mark the expected positions of myc-TRADD proteins (also compare with the lower panel). Apparent molecular weights are given in kiloDaltons.

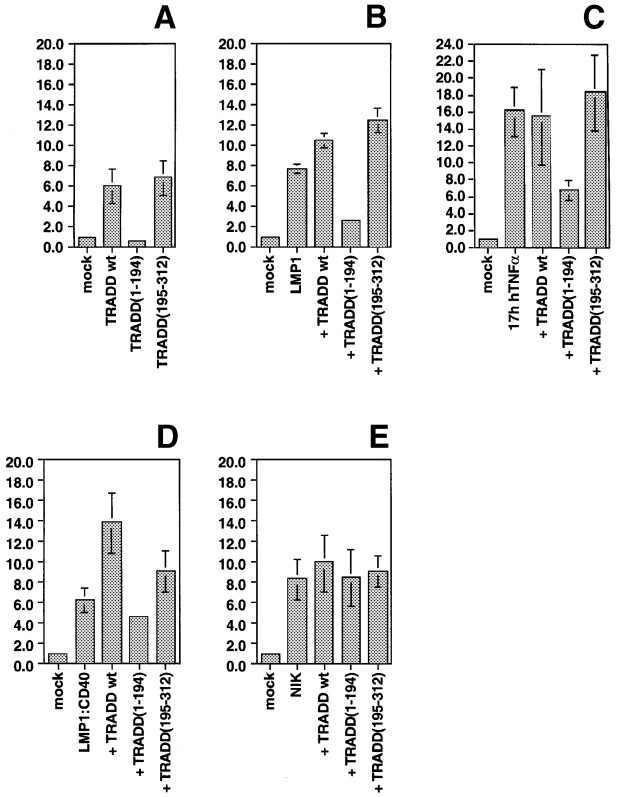

Fig. 4. TRADD(1–194) blocks NF-κB induction by LMP1 and TNFR1. Transient NF-κB reporter assays in 293 cells. The following expression vectors were co-transfected as indicated: 1 μg of pSV-LMP1, pSV-LMP1:CD40 or pcDNA3-NIK; 125 ng of pRK-myc-TRADD, pRK-myc-TRADD(1–194) or pRK-myc-TRADD(195–312). Mock-transfected controls were co-transfected with carrier DNA. To trigger TNFR1 activity, cells were stimulated with 20 ng/ml hTNFα for 17 h. pcDNA3-p35 (0.5 μg) was co-transfected to inhibit TRADD-induced apoptosis. Luciferase activities were corrected for transfection efficiencies (see Materials and methods). (**A**) Effects of TRADD wild-type and TRADD mutants on NF-κB activity. (B–E) The impact of TRADD wt and TRADD mutants on NF-κB activity induced by (**B**) LMP1, (**C**) TNFR1, (**D**) LMP1:CD40, a fusion protein of the LMP1 transmembrane domain with the C-terminal signaling domain of CD40, or (**E**) NIK. NF-κB activities are given as x-fold induction versus the mock-transfected controls (*y*-axis) and are mean values of at least three independent experiments.

lacking the death domain, TRADD(195–312) or TRADD(296–299A), as indicated. In TRADD(296–299A), amino acids 296–299 were changed into alanines. This mutant is impaired in its ability to bind to TNFR1 and does not induce NF-κB or apoptosis (Park and Baichwal, 1996). TRADD proteins were detected on immunoblots via the monoclonal anti-Myc tag antibody 9E10. As shown in Figure 3, lower panel, TRADD wt and all TRADD mutants were expressed at approximately equal levels in 293 cells as judged by immunoblotting analysis of cleared cell lysates. GST–LMP1 beads were used to precipitate TRADDs from these cell lysates (Figure 3, upper panel). As expected, full-length TRADD could be precipitated readily with GST–LMP1 beads but not with GST control beads. The signals seen in the GST control pull-downs stem from an unspecific binding of the antibodies used for immunostaining and the GST protein. TRADD(195–312), containing the TRADD death domain, did not co-precipitate with GST–LMP1 to detectable levels and, thus, did not bind efficiently to LMP1. This suggested to us that the TRADD death domain might not be necessary for an interaction between LMP1 and TRADD. Confirming this assumption, both TRADD(1–194) and TRADD(296–299A) bound very efficiently to the LMP1 C-terminus. Similar results could be obtained with pull-down experiments using *in vitro* translated TRADD proteins, making it likely that the binding of TRADD to LMP1 occurs via direct protein–protein interaction through TRADD's N-terminal part (data not shown). Co-immunoprecipitation experiments in 293 cells confirmed our previous results (data not shown). It is evident from these experiments (i) that mutation of the TRADD death domain does not impair its ability to interact with LMP1, and (ii) that the TRADD death domain does not bind efficiently to LMP1.

Our data provide the first evidence that TRADD can interact with receptor-like molecules independently of its death domain. The TRADD N-terminus (amino acids 1–194) is necessary and sufficient for TRADD's interaction with LMP1. It was now interesting to evaluate whether this 'upside-down' interaction of TRADD with LMP1 would have any consequences for the role of TRADD in LMP1 signal transduction to NF-κB and JNK1 as compared with its role in TNFR1 signaling.

A TRADD mutant lacking its death domain efficiently blocks LMP1 signaling to NF-κB

The TRADD death domain is absolutely required for NF-κB induction by TRADD (Hsu *et al.*, 1995; Park and Baichwal, 1996). Accordingly, TRADD(1–194) did not induce NF-κB in 293 cells (Figure 4A). As expected, both TRADD wild-type and the TRADD death domain, TRADD(195–312), induced NF-κB to similar levels

(Figure 4A). TRADD(1–194) lacks its NF-κB-inducing effector domain but still binds to LMP1. Therefore, we tested the TRADD(1–194) mutant for its potential to block LMP1 signaling to NF-κB in transient reporter assays in 293 cells. TRADD-induced apoptosis was prevented by co-transfection of the baculovirus p35 protein (Seshagiri and Miller, 1997). All TRADD proteins were expressed to equal levels in 293 cells (data not shown; Figure 3).

As shown in Figure 4B, TRADD(1–194) strongly interfered with NF-κB induction by LMP1. Transfection of 1 μg of pSV-LMP1 led to a 7.6-fold induction of NF-κB-dependent luciferase reporter gene activity. Co-transfection of 125 ng of pRK-myc-TRADD(1–194) reduced NF-κB induction by LMP1 to 2.6-fold. This result delivers the first formal proof that LMP1 induces NF-κB via a TRADD-dependent pathway and shows that the TRADD death domain is essential for LMP1 signaling to NF-κB. Since 293 cells express TNFR1 but not TNFR2 (Natoli *et al.*, 1997), 293 cells were stimulated with 20 ng/ml hTNFα for 17 h to study TRADD effects on TNFR1 signal transduction. As shown in Figure 4C, TRADD(1–194) also interferes with TNFR1 signaling to NF-κB. This is in agreement with previous results demonstrating that TNFR1 induction of NF-κB is dependent on TRADD (Park and Baichwal, 1996). In contrast, CD40 signal transduction to NF-κB is mediated by a direct interaction between TRAF2 and a PxQxT motif in the C-terminus of CD40, independently of TRADD (reviewed in Arch *et al.*, 1998). Therefore, TRADD(1–194) should not block CD40 signaling. Accordingly, co-expression of TRADD(1–194) only had a marginal effect on NF-κB induction by LMP1:CD40, demonstrating the specificity of the dominant-negative effect of this TRADD mutant in LMP1 and TNFR1 signaling (Figure 4D). LMP1:CD40 constitutes a fusion protein of the LMP1 transmembrane domain with the C-terminal signaling domain of CD40. LMP1:CD40 acts like a constitutively active CD40 (Gires *et al.*, 1997). TRADD(1–194) did not block NF-κB induction by NIK (Figure 4E). This result was expected and further excludes unspecific effects of TRADD(1–194) on NF-κB activity since NIK is supposed to work downstream of TRADD/TRAF2 in the signaling cascade to NF-κB (Arch *et al.*, 1998).

Both TRADD wt and the TRADD death domain (195–312) co-operated with LMP1 to induce NF-κB. In both cases, this effect was less than additive (Figure 4B). hTNFα- or NIK-triggered NF-κB activation was not augmented significantly by TRADD wt or TRADD(195–312) in this experimental setting (Figure 4C and E). In contrast, TRADD wt and LMP1:CD40 acted synergistically to induce NF-κB (Figure 4D), although CD40 does not signal via TRADD. TRADD- and CD40-triggered pathways seem to converge at a certain point in the signaling cascade to NF-κB, causing the observed synergism upon NF-κB activity. Thus, a synergism between two signaling molecules alone is not sufficient to postulate a direct signaling cascade involving both molecules. In summary, our results show that LMP1, like TNFR1, signals to NF-κB via TRADD. Moreover, the dominant-negative TRADD(1–194) should constitute a powerful tool to study further the role of TRADD in LMP1 signal transduction to JNK1 and NF-κB.

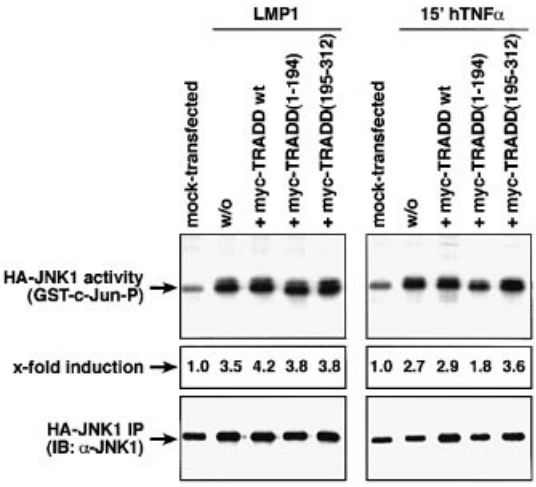

Fig. 5. TRADD(1–194) does not inhibit LMP1 activation of JNK1, but interferes with JNK1 induction by TNFR1. Transient HA-JNK1 assays in 293 cells. Left column: 1 μg of pSRα-HA-JNK1 was transfected either without (mock-transfected) or together with 0.5 μg of pSV-LMP1. Right column: instead of transfecting pSV-LMP1, 293 cells were treated with 20 ng/ml hTNFα for 15 min to trigger TNFR1 activity. As indicated, 125 ng of pRK-myc-TRADD, pRK-myc-TRADD(1–194) or pRK-myc-TRADD(195–312) were co-transfected. In addition, baculovirus p35 was expressed to block TRADD-induced apoptosis. Top panels: autoradiograph of GST–c-Jun *in vitro* phosphorylated by immunoprecipitated HA-JNK1. Middle panels: x-fold induction of HA-JNK1 kinase activities as quantitated by phosphoimager analysis. Bottom panels: immunoblotting analysis of the immunoprecipitated HA-JNK1 protein using the rabbit anti-JNK1 C-17 antibody.

Dominant-negative TRADD interferes with TNFR1 but not with LMP1 signaling to JNK1

The JNK1 and NF-κB activator sites of the CTAR2 domain are identical and both are overlapping with the TRADD-binding site of LMP1. TRADD(1–194), lacking its death domain, binds to LMP1 and blocks LMP1 signaling to NF-κB very efficiently. We were now interested to see whether TRADD(1–194) would also interfere with JNK1 induction by LMP1. For this purpose, we transiently transfected LMP1 together with TRADD wt and the TRADD(1–194) and TRADD(195–312) mutants in 293 cells and subsequently assayed for the activity of a co-transfected HA-JNK1. To study TRADD effects on TNFR1 signaling to JNK1, 293 cells were stimulated with 20 ng/ml hTNFα for 15 min prior to cell harvest. Again, baculovirus p35 was co-expressed to protect cells from TRADD-induced apoptosis. TRADD wt and mutants were expressed to approximately equal levels, as judged by immunoblotting analysis of cleared cell lysates performed prior to HA-JNK1 immunoprecipitation (data not shown and Figure 3). As shown in Figure 5, TRADD(1–194) did not block LMP1 signaling to JNK1. TRADD wt and the TRADD death domain, TRADD(195–312), had no significant impact on LMP1 signaling to JNK1 either. Thus, TRADD binding and induction of JNK1 by LMP1 appear not to be mutually exclusive. In contrast, TRADD(1–194) impaired JNK1 induction by TNFR1. hTNFα-induced JNK1 activity was reduced by ~50% after co-expression of TRADD(1–194). TRADD(195–312) slightly augmented JNK1 induction by hTNFα.

We conclude that, in contrast to TNFR1, LMP1 signaling to JNK1 does not involve the TRADD death domain.

TRADD(1–194) efficiently blocks LMP1 signaling to NF-κB (see Figure 4B) but not to JNK1. Apparently, the molecular mechanisms by which LMP1 induces JNK1 or NF-κB are already different at the level of the LMP1 signaling complex. Moreover, our results show that LMP1 and TNFR1 differ not only in the architecture of the receptor–TRADD complexes but also in the functional roles of the TRADD protein in signal transduction.

LMP1 signal transduction to JNK1 is not dependent on TRAF2

Sequences in the TRADD N-terminus (amino acids 1–169) are responsible for TRAF2 binding (Hsu *et al.*, 1996b). To our knowledge, TRAF2 is the only protein shown to interact physically with the TRADD N-terminus. A dominant-negative TRAF2 lacking its N-terminal RING-finger domain (amino acids 1–87) blocks signal transduction from LMP1, TNFR1 and TRADD to NF-κB (Rothe *et al.*, 1995; Devergne *et al.*, 1996; Hsu *et al.*, 1996b; Kaye *et al.*, 1996). Moreover, dominant-negative TRAF2(87–501) inhibits TNFR1 induction of JNK1 (Liu *et al.*, 1996; Natoli *et al.*, 1997). Since TRADD(1–194), which includes the TRAF2-binding sequences of TRADD, binds to LMP1, we tested the possiblity that TRAF2 might mediate LMP1 signaling to JNK1 (Figure 6). For that purpose, we performed transient HA-JNK1 kinase assays, in which we transfected 0.5 μg of pSV-LMP1 together with increasing amounts (0.1–1.0 μg) of the TRAF2(87–501) expression vector pRK-TRAF2(87–501). As a positive control, we assayed TRAF2(87–501) effects on JNK1 activity induced by TNFR1. Furthermore, LMP1:CD40 was included in the experiment. CD40 is known to signal to JNK1 via TRAF2 (Lee *et al.*, 1997). Both LMP1 and LMP1:CD40 are expressed from an identical vector background, and the activity of their signaling domains is only dependent on self-aggregation of the LMP1 transmembrane domain (Gires *et al.*, 1997). This experimental set-up allowed us to compare TRAF2(87–501) effects on the signaling domains of LMP1 and CD40 directly.

Whereas low amounts of co-transfected TRAF2(87–501) already significantly blocked TNFR1- and LMP1:CD40-induced JNK1 activity, TRAF2(87–501) had no inhibitory effect on JNK1 activation by LMP1 at all (Figure 6A). TRAF2(87–501) was expressed readily, as evaluated from immunoblotting analysis (Figure 6B). Most strikingly, 0.1 μg of pRK-TRAF2(87–501) sufficed to reduce LMP1:CD40-triggered JNK1 activity by ~90%. This demonstrates a pronounced dominant-negative effect of TRAF2(87–501) on the CD40 portion of LMP1:CD40. Notably, a large amount of pRK-TRAF2(87–501) had no impact on LMP1-triggered JNK1 activity. These results clearly show that LMP1, in contrast to TNFR1 and CD40, induces JNK1 via a pathway that does not involve TRAF2 directly. Moreover, these data confirm that the JNK1 and NF-κB signaling pathways originating at the CTAR2 domain of LMP1 bifurcate upstream of TRAF2 since LMP1 induces NF-κB activity via a TRAF2–NIK-dependent pathway (data not shown and Devergne *et al.*, 1996; Kaye *et al.*, 1996; Sylla *et al.*, 1998).

p21 Rho-like GTPases mediate TNFR1 but not LMP1 signaling to JNK1 in 293 cells

We have shown that the mechanisms by which LMP1 and TNFR1 induce JNK1 already differ at the level of the

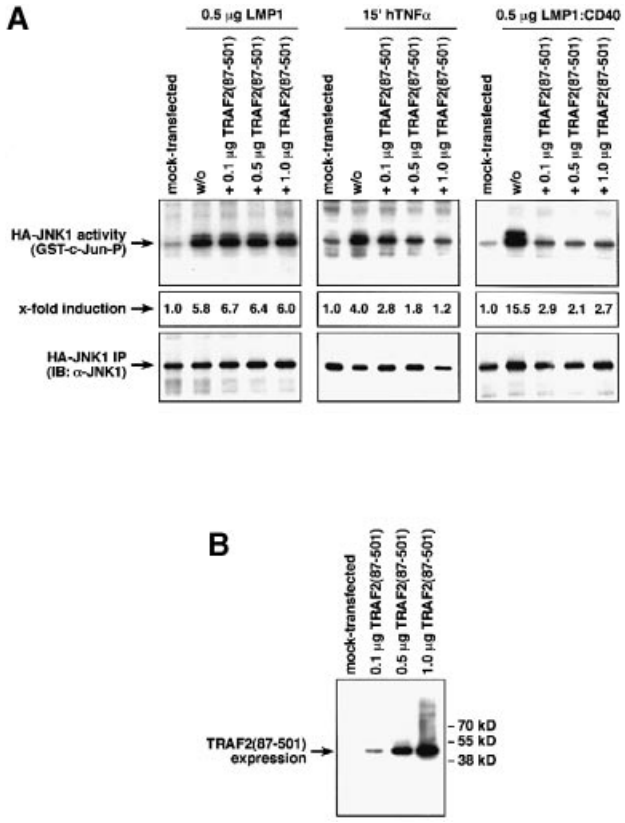

Fig. 6. Dominant-negative TRAF2 blocks TNFR1 and CD40 but not LMP1 signaling to JNK1. Transient HA-JNK1 assays in 293 cells. (**A**) 293 cells were transiently transfected with 1 μg of pSRα-HA-JNK1 together with 0.5 μg of pSV-LMP1 or pSV-LMP1:CD40, as indicated. TNFR1 activity was stimulated by treatment of cells with 20 ng/ml hTNFα for 15 min prior to cell harvest. The indicated amounts of pRK-TRAF2(87–501) expressing dominant-negative TRAF2 were co-transfected. At 24 h post-transfection, HA-JNK1 immunocomplex kinase assays were performed. Top panels: autoradiograph of GST–c-Jun *in vitro* phosphorylated by immunoprecipitated HA-JNK1. Middle panels: x-fold induction of HA-JNK1 kinase activities as quantitated by phosphoimager analysis. Bottom panels: immunoblotting analysis of the immunoprecipitated HA-JNK1 protein using the rabbit anti-JNK1 C-17 antibody. (**B**) TRAF2(87–501) expression in a representative co-transfection experiment as determined by immunoblotting analysis of total cell lysates using the mouse anti-TRAF2 antibody C-20. Apparent molecular weights are given in kiloDaltons.

pseudoreceptor or receptor signaling complexes, respectively. We were interested to see whether this observation would result in different downstream signaling pathways of LMP1 and TNFR1 to JNK1. So far, TNFR1 signal transduction to JNK1 is poorly defined. The p21 Rho-like GTPases Rac1 and Cdc42 induce the JNK1 pathway (Coso *et al.*, 1995; Minden *et al.*, 1995), and both GTPases are involved in JNK1 activation by TNFα, as was shown by transfection of dominant-negative Rac1 and Cdc42 mutants in COS-7 cells (Coso *et al.*, 1995). However, the role of Rac1 and Cdc42 in TNFα signal transduction appears to be cell type dependent, since Minden and colleagues found no evidence that a dominant-negative Rac1 blocks TNFα signaling to JNK1 in HeLa cells (Minden *et al.*, 1995).

We examined whether LMP1 or TNFR1 signal transduction to JNK1 is mediated by p21 Rho-like GTPases in 293 cells. For transient HA-JNK1 kinase assays, 293 cells

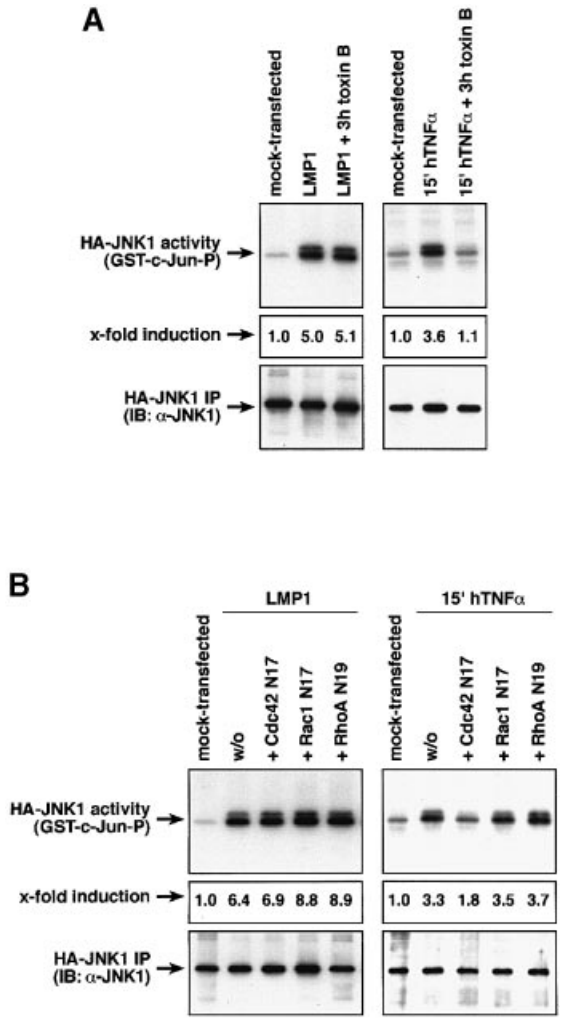

Fig. 7. TNFR1 induces JNK1 through a Cdc42-dependent pathway, whereas LMP1 signaling to JNK1 is independent of p21 Rho-like GTPases. Transient HA-JNK1 assays in 293 cells. (**A**) Toxin B, a specific inhibitor of p21 Rho-like GTPases, blocks TNFR1 but not LMP1 activation of JNK1. 293 cells were transfected with 1 μg of pSRα-HA-JNK1 together with 0.5 μg of pSV-LMP1 or carrier DNA. As indicated, cells were stimulated with 20 ng/ml hTNFα for 15 min to trigger TNFR1 activity and/or treated with 20 ng/ml toxin B for 3 h prior to cell harvest. At 24 h post-transfection, immunocomplex kinase assays were performed to determine HA-JNK1 activity.
(**B**) A dominant-negative Cdc42 mutant inhibits JNK1 induction by TNFR1 but not by LMP1. Transfections and hTNFα stimulations were performed as described in (A). In addition, 1.5 μg of pcDNA3-Cdc42N17, pcDNA3-Rac1N17 or pcDNA3-RhoAN19 were co-transfected as indicated. Top panels: autoradiograph of GST–c-Jun phosphorylation. Middle panels: x-fold induction of HA-JNK1 kinase activities. Bottom panels: immunoblotting analysis of the immunoprecipitated HA-JNK1 protein.

were either transfected with LMP1 or stimulated with 20 ng/ml hTNFα for 15 min to trigger TNFR1. To block p21 Rho-like GTPase activity, cells were treated with the *Clostridium difficile* toxin B for 3 h prior to cell harvest. Toxin B causes the specific glycosylation and inactivation of p21 Rho-like GTPases (Just *et al.*, 1995). Whereas TNFα stimulation of JNK1 activity could be blocked completely by toxin B treatment, toxin B had no effect on JNK1 induction by LMP1 (Figure 7A). These data suggested that TNFR1, in contrast to LMP1, induces JNK1 via p21 Rho-like GTPases in 293 cells. Apparently, LMP1 and TNFR1 downstream signaling pathways to JNK1 substantially differ from each other within the same cell. To confirm these results and to identify which of the p21 Rho-like GTPases actually mediates TNFR1 signaling, we examined the effects of dominant-negative mutants of Rac1 and Cdc42, Rac1N17 and Cdc42N17 (Coso *et al.*, 1995), on JNK1 induction by LMP1 and TNFR1. As a control, we further included a dominant-negative RhoA mutant, RhoAN19 (Coso *et al.*, 1995), which was expected not to block JNK1 activation. RhoA induces JNK1 very weakly but rather feeds into the serum response factor pathway (Coso *et al.*, 1995; Hill *et al.*, 1995). As shown in Figure 7B, none of the three dominant-negative mutants interfered with JNK1 induction by LMP1, confirming our previous results with toxin B treatment. In contrast, TNFR1 signaling was blocked specifically by co-transfection of Cdc42N17 but not of Rac1N17 or RhoAN19. Thus, in 293 cells, TNFR1 induces JNK1 via activation of Cdc42. We concluded that LMP1 and TNFR1 signaling cascades to JNK1 are not converging upstream of or at the level of p21 Rho-like GTPases. Our results clearly show that LMP1 makes use of substantially different mechanisms to induce JNK1 as compared with TNFR1.

Discussion

Here we have shown that LMP1 and TNFR1 signaling mechanisms differ substantially from each other. This is especially interesting since both molecules recruit TRADD and TRAFs to exert their biological effects in the cell. However, LMP1 is a transforming viral oncogene, whereas TNFR1 in many cases signals cell death. TNFα-induced trimerization of TNFR1 results in the binding of TRADD via interaction of the death domains of both molecules (Hsu *et al.*, 1996b). This interaction initiates the formation of a receptor signaling complex responsible for JNK1, NF-κB and apoptosis induction (Hsu *et al.*, 1995, 1996b; Liu *et al.*, 1996). TRADD mediates the entry of TRAF2 into the complex (Hsu *et al.*, 1996b). TRAF2 is critically involved in induction of JNK1 and NF-κB by TNFR1. Thus, both pathways bifurcate downstream of TRAF2 in TNFR1 signal transduction (Hsu *et al.*, 1996b; Liu *et al.*, 1996). Here we present evidence that the LMP1 CTAR2-triggered signaling cascades to JNK1 and NF-κB diverge upstream of TRAF2, either at the level of TRADD or at the level of LMP1 itself. In contrast to TNFR1, LMP1 signaling to JNK1 appears to be independent of the TRADD death domain and TRAF2. Moreover, LMP1 activation of JNK1 does not involve p21 Rho-like GTPases, confirming that LMP1 and TNFR1 target JNK1 via different mechanisms (Figure 8).

We have identified the JAR of the LMP1 molecule comprising amino acids at positions 379–384. This sub-domain overlaps with the NF-κB-activating region and the TRADD-binding domain of the LMP1 CTAR2 domain. This result led us to investigate the role of TRADD and TRAF2 in LMP1 signaling to JNK1 and NF-κB. Using biochemical approaches, we found that a functional TRADD death domain is not necessary for TRADD binding to LMP1. LMP1, which does not contain a death domain itself, complexes with the TRADD N-terminus but not with the TRADD death domain. This type of inverse binding between a receptor or receptor-like molecule and TRADD is a novel 'upside-down' interaction of

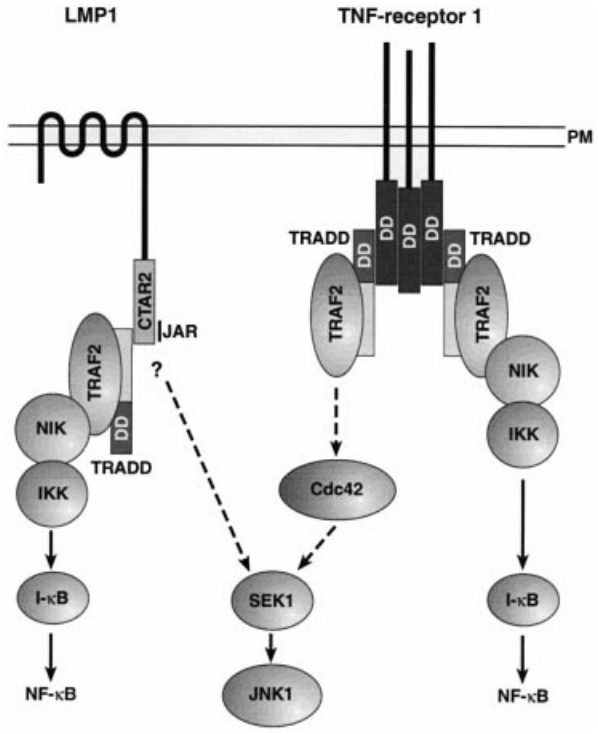

Fig. 8. Model of LMP1 CTAR2 and TNFR1 signal transduction to JNK1 and NF-κB. For detailed explanations, see the text. Abbrevations not defined in the text are: DD, death domain; I-κB, inhibitor of NF-κB; IKK, I-κB kinase; PM, plasma membrane; SEK1, stress-enhanced kinase 1. Note the 'upside-down' interaction of TRADD with LMP1 as compared with TNFR1 and the different pathways triggered by LMP1 and TNFR1 leading to JNK1 induction. In contrast to TNFR1, JNK1 and NF-κB pathways originating at the CTAR2 domain of LMP1 bifurcate upstream of TRAF2.

TRADD. Our results show that the LMP1–TRADD and TNFR1–TRADD signaling complexes are of different structure and indicate potential differences in TRADD-mediated signaling between LMP1 and TNFR1. We performed TRADD-binding assays under stringent conditions (150 mM NaCl) to detect specific and efficient binding of TRADD and TRADD mutants to LMP1. In our assays, we could not see any detectable levels of complex formation between the TRADD death domain and the LMP1 C-terminus. Amino acids 195–312 exactly comprising the TRADD death domain have been identified previously as the LMP1-docking site of TRADD in a yeast two-hybrid screen (Izumi and Kieff, 1997). Possibly, a weak interaction between LMP1 and the TRADD death domain that could not be detected under our experimental conditions might account for the positive result of the two-hybrid screen. From our data, it is clear, however, that the TRADD N-terminus binds to LMP1 (i) with much higher affinity than the TRADD death domain and (ii) independently from the TRADD death domain. What implications could the 'upside-down' interaction have for the role of TRADD in LMP1 function? The death domain is responsible for TRADD's binding to other death domain proteins (Yuan, 1997). The 'upside-down' interaction could, for example, mediate an interaction of LMP1 with other death domain receptors in the plasma membrane to allow cross-talk to different receptors or competition.

The death domain is necessary and sufficient for NF-κB induction by TRADD (Hsu *et al.*, 1995, 1996b; Park and Baichwal, 1996) but is not essential for LMP1 binding. Thus, we could identify TRADD(1–194) as a powerful dominant-negative mutant efficiently blocking LMP1 signaling to NF-κB. TRADD(1–194) reduced LMP1 wild-type-triggered NF-κB activity by ~70%. Since ~70% of the total capacity of LMP1 to induce NF-κB is contributed by CTAR2 (Huen *et al.*, 1995; Mitchell and Sugden, 1995), TRADD(1–194) apparently blocks the CTAR2-derived NF-κB activity entirely. This is the first formal prove that LMP1 signals to NF-κB via TRADD. Previous reports concluded from a synergism between LMP1 and TRADD upon NF-κB activity that there was a direct signaling pathway involving LMP1 and TRADD. However, TRADD-independent NF-κB inducers such as CD40 also co-operate with TRADD to induce NF-κB (see Figure 4D). A specifically acting dominant-negative TRADD mutant delivers additional and conclusive evidence for a TRADD-dependent pathway from LMP1 to NF-κB. As expected, TRADD is also involved in TNFR1-triggered induction of NF-κB since TRADD(1–194) did impair TNFR1-induced NF-κB activity. How can the effect of dominant-negative TRADD(1–194) on TNFR1 signaling be explained? TRADD(1–194) cannot bind to TNFR1 (Hsu *et al.*, 1995) and, thus, should not be able to block the TNFR1 docking sites for wild-type TRADD molecules. It is most likely that TRADD(1–194) sequesters TRAF2 molecules and/or other factors necessary for TRADD signaling from the cytoplasm, making them unavailable for the TNFR1 signaling complex. Moreover, TRADD(1–194) could disturb the integrity of the TNFR1 signaling complex by interacting with molecules of the complex. Such dominant-negative mechanisms should not work as efficiently as a direct block of the receptor by mutant TRADD molecules. Accordingly, the dominant-negative effect of TRADD(1–194) was still remarkable but less prominent with TNFR1 signaling as compared with NF-κB induction by LMP1 (see Figures 4 and 5).

Here we have shown that TRADD(1–194) blocks both LMP1 and TNFR1 signaling to NF-κB. A still unexplained discrepancy in the literature is that, whereas the TRAF2 binding site lies in the TRADD N-terminus, the TRADD death domain alone induces NF-κB (Yuan, 1997). In fact, the death domain is both necessary and sufficient for NF-κB induction by TRADD (Hsu *et al.*, 1995; Park and Baichwal, 1996). TRAF2 binds to TRADD, and dominant-negative TRAF2(87–501) interferes with LMP1- and TNFR1-induced NF-κB activity (Devergne *et al.*, 1996; Hsu *et al.*, 1996b; Kaye *et al.*, 1996). TRAF2(87–501) also blocks TRADD induction of NF-κB (data not shown), placing TRAF2 downstream of TRADD in the signaling cascade from TRADD to NF-κB. NIK directly binds to TRAF2, and a dominant-negative mutant of NIK interferes with LMP1 and TNFR1 induction of NF-κB (Malinin *et al.*, 1997; Sylla *et al.*, 1998). Based on the above-cited results and our own data, we postulate a hierarchical signaling pathway triggered by the CTAR2 domain of LMP1 involving TRADD→TRAF2→NIK (Figure 8). Apparently, TNFR1 signals to NF-κB via the same signaling cascade as LMP1, although the TNFR1–TRADD complex exhibits a different molecular architecture from that of the LMP1–TRADD complex.

Whereas TRADD(1–194) is a potent inhibitor of LMP1-induced NF-κB activity, this TRADD mutant had no effect

on JNK1 induction by LMP1. These results strongly suggest that LMP1 induces JNK1 and NF-κB via different mechanisms. In contrast, TNFR1 signaling to NF-κB and to JNK1 can be partially blocked by TRADD(1–194). We conclude from these data (i) that the JNK1 and NF-κB signaling pathways originating at CTAR2 bifurcate either at the level of TRADD or upstream at the level of the JAR subdomain in CTAR2 and (ii) that LMP1 and TNFR1 induce JNK1 via different molecular mechanisms. Furthermore, the TRADD death domain and, thus, molecules binding to the TRADD death domain such as RIP, most likely do not account for JNK1 activation by LMP1. Since TNFR1 signal transduction to JNK1 is mediated by TRAF2 (Natoli *et al.*, 1997), we examined whether LMP1 signaling to JNK1 might involve TRAF2. As expected, low amounts of TRAF2(87–501) efficiently blocked JNK1 induction by TNFR1 and LMP1:CD40. TRAF2(87–501) had no effect on LMP1 signaling to JNK1, further substantiating our conclusions that LMP1 and TNFR1 make use of different mechanisms to induce JNK1 already at the level of the receptor signaling complexes. From our data, we cannot fully exclude the possibility that LMP1 signaling to JNK1 is mediated via the TRADD N-terminus. In this case, however, a new TRAF2-independent pathway targeting JNK1 that is initiated at the TRADD N-terminus must be postulated. So far, there is no hint of such a pathway, making it likely that bifurcation of the NF-κB and JNK1 pathways takes place at the JAR motif. Currently, we are trying to identify candidate proteins binding to CTAR2 that could account for JNK1 induction by LMP1. We could demonstrate that LMP1 and TNFR1 signal to JNK1 via divergent pathways. Differences in the involvement of the p21 Rho-like GTPase Cdc42 in signal transduction of LMP1 and TNFR1 to JNK1 within the same cell line further support our conclusions.

In summary, we have shown that the topologies of the LMP1 and TNFR1 signaling complexes differ from each other, although both LMP1 and TNFR1 directly interact with TRADD. This observation correlates with substantial differences between TNFR1 and LMP1 signal transduction to JNK1 but not to NF-κB. Moreover, JNK1 and NF-κB pathways originating at the CTAR2 domain of LMP1 appear to bifurcate upstream of TRAF2 and differ in the molecular role of TRADD. Our results might help to understand why LMP1 and TNFR1 elicit different cellular responses although working through similar signaling mediators.

Materials and methods

Plasmids

pSV-LMP1 and pSV-LMP1Δ212–231 have been described previously (Kieser *et al.*, 1997). To generate pSV-LMP1(PQT→AAA), an *Xho*I–*Bpu*11O2I fragment of pSG5-LMP1.AAA (Floettmann *et al.*, 1998) was cloned into the pSV-LMP1 background. pSV-LMP1(PQT→AAA/Y384G) and pSV-LMP1(Y384G) were cloned by PCR approaches from pSV-LMP1(PQT→AAA) or pSV-LMP1, respectively. Primer sequences are available upon request. To generate pSV-LMP1Δ194–386, an *Xho*I–*Bpu*11O2I fragment of pSG5-LMP1Δ[194–386] (Huen *et al.*, 1995) was cloned into the pSV-LMP1 background. The pSG5-LMP1 vector and the pSG5-LMP1-based vectors coding for the LMP1 mutants H377G, P379G, V380G, Q381G, S383G, Y384G, Y385G and D386G have been described (Floettmann and Rowe, 1997). The pRK-myc-TRADD expression vector has been described (Hsu *et al.*, 1995). The expression vectors pRK-myc-TRADD(1–194), pRK-myc-TRADD(195–312) and pRK-myc-TRADD(296–299A) have been cloned by PCR approaches from pRK-myc-TRADD. Primer sequences are available upon request. The expression vectors pSRα-HA-JNK1 (Minden *et al.*, 1994), pcDNA3-NIK (Malinin *et al.*, 1997), pcDNA3-p35 (Seshagiri and Miller, 1997), pSV-LMP1:CD40 (Gires *et al.*, 1997), pRK-TRAF2(87–501) (Hsu *et al.*, 1996b; Natoli *et al.*, 1997), pcDNA3-Cdc42N17, pcDNA3-Rac1N17 and pcDNA3-RhoAN19 (Coso *et al.*, 1995) have been described. pGEX-LMP1 coding for GST–LMP1 (amino acids 181–386 of LMP1) has been described (Sandberg *et al.*, 1997). pGEX-2T is commercially available (Pharmacia). The NF-κB reporter plasmid 3X-κB-L has been described (Mitchell and Sugden, 1995) and the β-galactosidase reporter CMVβGal is commercially available (Clontech).

Cell culture methods

293 human embryonic kidney cells were grown in full medium containing 10% fetal calf serum (FCS; Gibco). For transfections, cells were grown to subconfluence in 6-well plates (Nunc). As indicated, 293 cells were transfected with 2–3 μg of DNA using the Lipofectamine reagent (Gibco) according to the manufacturer's protocol. Total amounts of transfected DNA were adjusted using salmon testes DNA (Sigma) as a carrier. After transfection, cells were grown in medium containing 1% FCS for 24 h to down-regulate serum-activated signaling pathways and treated as indicated in the figure legends or in the text. Subsequently, immunocomplex kinase assays, pull-down assays or reporter gene assays were performed. For triggering of TNFR1 activity, 293 cells were treated with 20 ng/ml of recombinant human TNFα (Boehringer Mannheim) for the time periods indicated. In order to block p21 Rho-like GTPases, cells were treated with 20 ng/ml of *C.difficile* toxin B (Just *et al.*, 1995) for 3 h prior to cell harvest.

Immunocomplex kinase assays, immunoblotting and antibodies

Cells were treated as described in the figure legends and in the previous section. Subsequently, cells were lysed in TBST buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM β-glycerophosphate, 0.5 mM sodium pyrophosphate, 0.5 mM sodium fluoride, 0.5 mM sodium molybdate, 0.5 mM sodium orthovanadate). Cleared lysates were incubated overnight with the monoclonal anti-HA antibody 12CA5 (Boehringer Mannheim), immobilized to protein G–Sepharose beads (Pharmacia), to immunoprecipitate HA-JNK1. Beads were washed twice with TBST and once with kinase reaction buffer [20 mM Tris–HCl, pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, 1 μM dithiothreitol (DTT), 2 μM ATP]. *In vitro* kinase reactions to assay the activity of immunoprecipitated HA-JNK1 were performed in kinase reaction buffer in the presence of 10 μCi of [γ-^{32}P]ATP per reaction sample using purified GST–c-Jun as a substrate (Kieser *et al.*, 1997). Kinase reactions or total cell lysates were separated by SDS–PAGE and blotted onto Hybond-C membranes (Amersham). Kinase reactions were analyzed by autoradiography and phosphoimager scanning. As indicated, the following primary antibodies were used for immunostaining of blots: rabbit anti-JNK1 C-17 (Santa Cruz Biotech.), mouse anti-LMP1 CS1-4 (Dako), mouse anti-Myc epitope 9E10 (Boehringer Mannheim), mouse anti-HA epitope 12CA5 and mouse anti-TRAF2 C-20 (Santa Cruz Biotech.). Immunoblots were analyzed using horseradish peroxidase (HRP)-coupled secondary antibodies (Dianova) and the ECL reagent (Amersham).

TRADD pull-down assays

293 cells were transiently transfected with 125 ng of myc-TRADD expression vectors as indicated. At 24 h post-transfection, cells were lysed in TBST (see above). Subsequently, cleared cell lysates were incubated overnight with GST–LMP1 or GST, expressed in *Escherichia coli* and immobilized to glutathione–Sepharose beads (Pharmacia), to co-precipitate myc-TRADD proteins. Beads were washed three times with TBST, and co-precipitated myc-TRADD proteins were analyzed by immunoblotting (see above) using the mouse anti-Myc epitope antibody 9E10.

Reporter assays

293 cells were transfected and treated as described in the legend to Figure 4 and in 'Cell culture methods'. To assay NF-κB activity, 5 ng of the NF-κB reporter 3×-κB-L were co-transfected per well of a 6-well plate. In addition, 50 ng of CMVβGal were co-transfected to standardize assays for transfection efficiencies. At 24 h post-transfection, cells were lysed in luciferase lysis buffer (100 mM KP_i, pH 7.8, 1 mM DTT, 1% Triton X-100). Luciferase and β-galactosidase activities were measured in cleared lysates. Luciferase assays were performed in luciferase assay

buffer [25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 5 mM ATP and 15 μg of D(–) luciferin (Boehringer Mannheim) per sample]. β-Galactosidase activity was measured using the Galacton/Emerald system (Tropix). Luciferase activities were corrected for β-galactosidase activities. NF-κB activation was calculated as fold induction as compared with mock-transfected controls.

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