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Interaction of human TNF and β2-microglobulin with Tanapox virus-encoded TNF inhibitor, TPV-2L

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ABSTRACT

Tanapox virus (TPV) encodes and expresses a secreted TNF-binding protein, TPV-2L or gp38, that displays inhibitory properties against TNF from diverse mammalian species, including human, monkey, canine and rabbit. TPV-2L also has sequence similarity with the MHC-class I heavy chain and interacts differently with human TNF as compared to the known cellular TNF receptors or any of the known virus-encoded TNF receptor homologs derived from many poxviruses. In order to determine the TNF binding region in TPV-2L, various TPV-2L C-terminal truncations and internal deletions were created and the muteins were expressed using recombinant baculovirus vectors. C-terminal deletions from TPV-2L resulted in reduced binding affinity for human TNF and specific mutants of TNF that discriminate between TNF-R1 and TNF-R2. However, deletion of C-terminal 42 amino acid residues totally abolished the binding of human TNF and its mutants. Removal of any of the predicted internal domains resulted in a mutant TPV-2L protein incapable of binding to human TNF. Deletion of C-terminal residues also affected the ability of TPV-2L to block TNF-induced cellular cytotoxicity. In addition to TNF, TPV-2L can also form complexes with human β2-microglobulin to form a novel macromolecular complex. In summary, the TPV-2L protein is a *bona fide* MHC-1 heavy chain family member that binds and inhibits human TNF in a fashion very distinct from other known poxvirus-encoded TNF inhibitors, and also can form a novel complex with the human MHC-1 light chain, β2-microglobulin.

Introduction

Tumor necrosis factor (TNF), the prototype member of the TNF superfamily of cytokines is an important immune regulator in orchestrating early defense against viral infection (Benedict, 2003; Benedict et al., 2003; Rahman and McFadden, 2006). The active pro-inflammatory ligand is predominantly synthesized as a cell surface homotrimer that can be cleaved and secreted from activated macrophages and monocytes. The biological effects of TNF, mainly to upregulate inflammation and apoptosis, are mediated by binding to two receptors, TNF-R1 or TNF-R2, members of the TNF-R superfamily of proteins (Aggarwal, 2003; Hehlgans and Pfeffer, 2005). The interaction of TNF with any of the TNF-Rs activates a signaling cascade leading to the triggering of downstream events that contribute to the host anti-viral responses. As a counter-protection measure, many viruses have acquired diverse strategies to manipulate TNF or TNF-mediated responses. Some of the known anti-TNF viral immunomodulatory molecules can directly bind and inhibit the function of TNF ligand whereas others modulate the TNF and TNF-R signaling pathway (s) (Benedict et al., 2003; Rahman and McFadden, 2006).

Poxviruses encode a plethora of immunomodulators which are important for evasion from host immune system (Nazarian and McFadden, 2006; Seet et al., 2003). Among them, TNF inhibitors are particularly vital for virus survival and most poxviruses have evolved mechanisms to inhibit TNF and TNF-mediated signaling. For example, poxviruses commonly express TNF modulators that can bind and sequester extracellular TNF and inhibit TNF-R activation (Rahman and McFadden, 2006). Two distinct types of poxvirus-encoded TNF inhibitors have been identified so far, one group most closely resembles secreted versions of the mammalian cell surface TNF-Rs, termed vTNF-Rs, and the other group, exemplified by TPV-2L, more closely resembles the mammalian MHC class I heavy chain, termed vTNF-BPs.

Viral TNF inhibitors that resemble TNF-Rs display diverse ligand binding specificities within the mammalian cytokines and chemokines (Rahman and McFadden, 2006). vTNF-Rs can be further subdivided into T2-like inhibitors encoded by the Leporipoxviruses and the Crm (cytokine response modifier) like inhibitors encoded by the orthopoxviruses (Cunnion, 1999). The vTNF-Rs retain characteristic CRDs (Cysteine rich domains) like their mammalian TNF-R counterparts, but do not maintain a transmembrane anchoring domain found in the cellular receptors. Myxoma virus-encoded

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protein M-T2 also possesses characteristic CRDs and not only does the secreted viral protein bind and inhibit rabbit TNF, but the intracellular version of the viral protein can also inhibit apoptosis in virus-infected lymphocytes (Schreiber et al., 1997). Further studies have found that M-T2 also harbors a PLAD (PreLigand Assembly Domain) like-domain present in cellular TNF-Rs (Sedger et al., 2006). The PLAD domain of M-T2 can inhibit human TNF-R-mediated cell death by interaction with the PLAD domains of both human TNF-R1 and TNF-R2 (Sedger et al., 2006). Crm-like TNF inhibitors namely CrmB, CrmC, CrmD and CrmE also possess multiple ligand binding properties. In case of CrmB and CrmD the C-terminal domain (CTD) exhibits chemokine binding properties, while the N-terminal domain retains the TNF binding/inhibition properties (Alejo et al., 2006). In contrast, the shorter CrmC and CrmE proteins which lacks CTD possess only TNF binding properties. Among the orthopoxviruses, Cowpox virus (CPXV) encodes all four Crm paralogues (Hu et al., 1994; Loparev et al., 1998; Saraiva and Alcami, 2001; Smith et al., 1996). In contrast, Ectromelia virus encodes only CrmD (Smith and Alcami, 2000) and variola and monkeypox viruses encode CrmB only. The solved crystal structure of CrmE from vaccinia virus strain Lister suggests that vTNF-Rs most closely resemble mammalian TNF-R2 (Graham et al., 2007).

Some poxvirus family members, such as the Yatapoxviruses and swine poxvirus, encode a unique class of TNF inhibitors, vTNF-BP, which are very distinct from the vTNF-Rs or other cellular TNF-R family members. Instead, the viral TNF-BPs share distant sequence similarities with MHC class I heavy chain. The first vTNF-BP was identified from Tanapox virus (TPV), and is called gp38 or TPV-2L, encoded by ORF 2L (Brunetti et al., 2003). Expressed TPV-2L protein binds and inhibits human, monkey and canine TNF with very high affinity (Brunetti et al., 2003; Rahman et al., 2006). It was also reported that the supernatants of TPV-infected cells contain protein (s) that neutralize the biological activity of IL-2, IL-5, IFN-γ and TNF. The activity responsible for this inhibition was originally designated as gp38, based on its molecular mass of 38 kDa (Essani et al., 1994; Paulose et al., 1998). However, the purified TPV-2L showed molecular mass of about 45 kDa, inhibited only TNF and lacked any detectable binding to IL-2, IL-5 and IFN-γ. One ortholog of this protein, YMTV-2L, encoded by Yaba monkey tumor virus (YMTV) can also bind and inhibit human and monkey TNF (Rahman et al., 2006). On the other hand, the vTNF-BP protein from Swinepox virus exclusively binds and inhibits only porcine TNF (Rahman et al., 2006). The vTNF-BPs were shown to be biochemically distinct from the cellular TNF-Rs in terms of binding to human TNF (hTNF). As shown with TPV-2L, hTNF mutants that selectively bind to either TNF-R1 or TNF-R2 can still bind to TPV-2L, but with different affinities. This suggests that TNF and TPV-2L interaction domains for binding to the cellular TNF-Rs are unique.

Here, we have characterized the human TNF binding properties of TPV-2L by creating TPV-2L C-terminal and internal deletions. Our results indicate that, except for the C-terminal 28 amino acids, the bulk of the TPV-2L protein is critical for hTNF binding. TPV-2L also can interact with human beta2-micoglobulin (hβ2m), in a fashion similar to the cellular MHC class I heavy chain molecule, to form a novel viral-host protein complex that we propose will have novel immunomodulatory properties.

Results and discussion

Expression of TPV-2L deletion constructs using the baculovirus expression system

TPV-2L and other members of vTNF-BPs exhibit distant similarity with the α1, α2 and α3 domains of cellular MHC class I heavy chain molecule. TPV-2L is a 338 amino acids long protein and has four potential N-linked glycosylation sites (N22, N68, N99 and N213) but no predicted O-linked glycosylation sites. This results in the expressed TPV-2L protein having a higher observed molecular mass (approximately 45 kDa) than calculated by just the amino-acid sequence (38 kDa). The presence of N-linked glycosylation sites in TPV-2L has been confirmed by treating purified protein with Endo H and PNGase F (data not shown). To assess any predicted protein–protein interaction domain(s) that might interact with TNF or possibly other proteins, we have created a series of C-terminal and internal deletions in TPV-2L (Figs. 1A and B). Since TPV-2L is expressed as a secreted glycoprotein, we left the N-terminal secretion signal sequence intact in all the deletion constructs. The deletions are named according to the first amino acid codon deleted within the truncated protein and encompass deletions ranging from 143 amino acids (TPV-2LΔK196) to the deletion of only the C-terminal 11 amino acids (TPV-2L-ΔE328). The C-terminal deletion TPV-2L proteins was expressed as Myc-His tagged versions and also expressed using recombinant baculoviruses. The expression of these TPV-2L deletion recombinant proteins was confirmed in the supernatants of the baculo-infected Sf21 cells. As can be seen in Fig. 2A, the expression level of secreted protein was different for each of the different C-terminal deletion constructs, when equal amounts of supernatant were loaded. The native TPV-2L and all the deletion constructs migrated with an apparent molecular mass larger than that predicted from the amino acid sequence, suggesting that these proteins were all glycosylated to some extent. Deletion of 143 amino acids from the C-terminus (TPV-2LΔK196), which removes the fourth predicted glycosylation site, did not affect the secretion of the protein (Fig. 2A, lane 1). This indicates that at least this CHO-modification site does not have a major role in TPV-2L expression level or secretion. However, when we pre-treated the cells with tunicamycin, an inhibitor of N-linked glycosylation, the expression of the native as well as the truncated TPV-2L proteins were all inhibited (data not shown).

In addition to the C-terminal deletions, several internal deletions of TPV-2L were created; they are outlined in Fig. 1B. They were also expressed as Myc-His tagged using baculovirus expression system. The expression of the truncated proteins was checked from the supernatant of the infected Sf21 cells. However, in some cases the mutant TPV-2L protein secretion levels were severely reduced (Fig. 2B). Deletion between K196 and C297 residues did not affect the secretion of expressed proteins and the level of secreted protein was as robust as the native TPV-2L (Fig. 2B, lane 7). This further supports the observation that the predicted fourth C-terminal glycosylation site

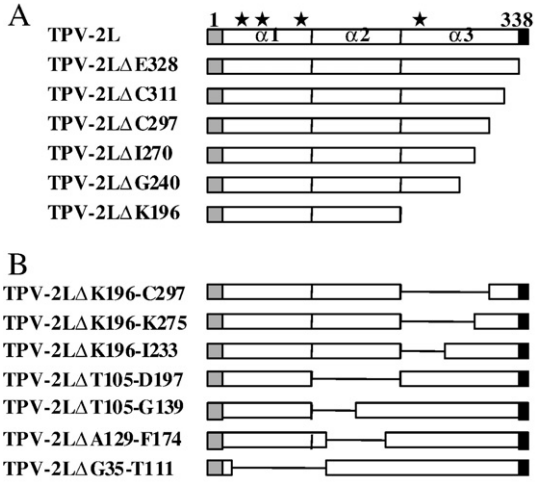

Fig. 1. Schematic representation of the TPV-2L constructs. (A) TPV-2L native protein (1–338 amino acids) is shown with three hypothetical domains α1, α2 and α3; stars showing the predicted N-glycosylation sites; grey boxes indicating the secretory leader sequence; black boxes indicating the C-terminal acidic domain. The different C-terminal deletion proteins are shown aligned with TPV-2L sequence, with rectangular boxes terminating at the point of each truncation. (B) Schematic representation of the TPV-2L internal deletion proteins; grey boxes indicating the secretory leader sequence; black boxes indicating the C-terminal acidic domain.

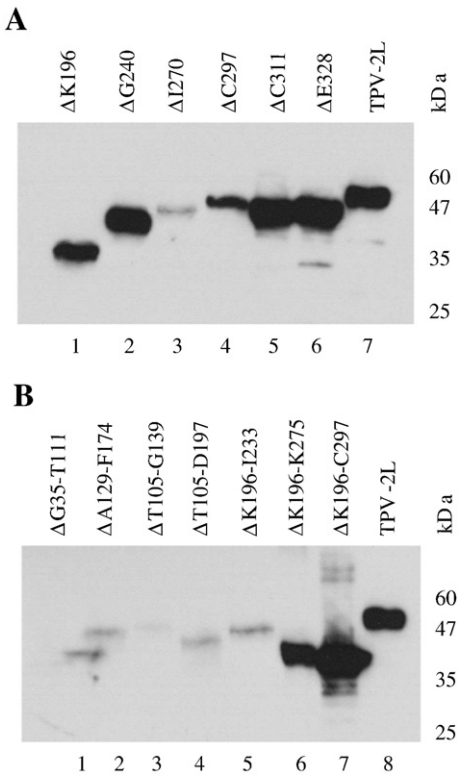

Fig. 2. Expression of TPV-2L deletion proteins. The baculovirus expressed Myc-His tagged TPV-2L C-terminal deletion proteins (A) and internal deletion proteins (B) from the infected Sf21 cell supernatants. Proteins were separated on a 10% polyacrylamide gel and transferred to a PVDF membrane that subsequently was probed with mouse anti-Myc antibody and HRP conjugated Goat anti-mouse antibody for detection. Lanes are as marked and molecular size markers are indicated in kDa.

at 213 amino acid is not important for TPV-2L expression or secretion. However, deletion of N-terminal TPV-2L residues which cover the predicted α2 and α3 domains severely reduced the secretion levels of the expressed proteins (Fig. 2B, lanes 1–4).

Kinetic and affinity analysis of TPV-2L deletions with hTNF and hTNF mutants

To test whether the different TPV-2L deletion proteins can interact with human TNF, screening was done by the Surface Plasmon Resonance (SPR) method using Biacore X. The mutant myc-his tagged proteins expressed in the baculovirus infected cell supernatant were partially purified using metal affinity resin. The proteins were then individually immobilized at high density (~2000–3000 RU) on NTA sensor chips. Human TNF ligand was injected over the control (flow cell 1) and immobilized protein surface (flow cell 2) in the chip. Among various C-terminal deletion proteins the mutants which lack 11 (TPV2L-ΔE328) and 28 (TPV2L-ΔC311) amino acids, respectively, retained efficient TNF binding properties (Figs. 3B and C). However, further deletion of 14 amino acids (TPV2L-ΔC297) essentially abolished the TNF binding activity of the TPV-2L protein. None of the other C-terminal deletion proteins bound to TPV-2L, although they expressed and secreted as the native proteins (data not shown). None of the internal 2L deletion proteins were able to bind human TNF (data not shown). This suggests that only the C-terminal 28 amino acid residues of TPV-2L protein are dispensible for TNF binding.

The TPV-2L deletion proteins which bound to human TNF were further tested for their individual kinetic binding analysis. The proteins were further purified using metal affinity column and independently immobilized at low densities (~500 RU) on CM5 chips for kinetic and affinity analysis. Different concentrations of human TNF and human TNF mutants were applied on the immobilized mutant TPV-2L proteins. The TNF mutants used in this study are R32W-S86T (hTNF32–86) and D143N-A145R (hTNF143–145), which have different binding affinities for TPV-2L and a related protein YMTV-2L from YMTV (Rahman et al., 2006). The mutations are located in or near the three loops (positions 30–36, 84–88, and 138–150) important for interaction with TNF-Rs. Previous studies have demonstrated that human TNF mutants R32W-S86T selectively bound only to TNF-R1, whereas D143N-A145R bound only to TNF-R2 (Loetscher et al., 1993; Van Ostade et al., 1994). For kinetic analysis, following an association period of 120 s, running buffer HBS-EP was injected to monitor the dissociation phase of binding (Figs. 3B and C). Kinetic analysis was performed as described before (Rahman et al., 2006). Table 1 summarizes the kinetic binding parameters of TPV-2L deletions with human TNF and TNF mutants. Deletion of 11 and 28 amino acid residues from C-terminus of TPV-2L had little effect on interaction with native human TNF, as both the deletion proteins bound to hTNF with high affinity. However, deletion of these residues severely reduced the binding and interaction with hTNF mutants. None of the hTNF mutants bound to TPV-2L mutants which did not interact with native hTNF. As reported before, hTNF32-86 had significantly reduced (14 fold) binding affinity with TPV-2L than native TNF (Rahman et al., 2006). This reduced binding was further potentiated by deletion of TPV-2L C-terminal residues. In case of TPV-

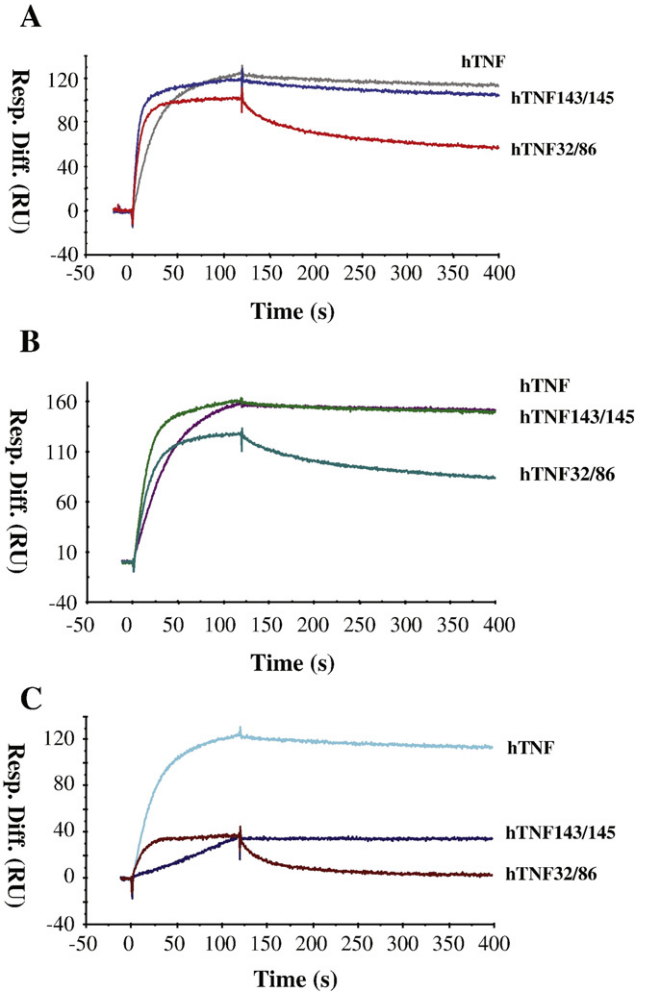

Fig. 3. Binding of TPV-2L mutants with hTNF and hTNF mutants. Over the immobilized TPV-2L mutant proteins sensor chip surface was passed 100 μl of hTNF and hTNF mutants. Sensograms showing the binding of hTNF and hTNF mutants, hTNF32/86 and hTNF143/145 (50 nM each) to TPV-2L (A), TPV-2LΔE328 (B) and TPV-2LΔC311 (C) analyzed by SPR (BIAcore X).

Table 1
Kinetic binding parameters and affinity constants of TPV-2L mutants to hTNF and hTNF mutants

TPV-2L/mutants	hTNF/mutants	*Ka* (1/Ms)	*Kd* (1/s)	K_D, nM	Chi^2
TPV-2L	hTNF	5.26e^6	2.25e^{-4}	0.043	1.6
	hTNF32-86	3.52e^6	2.11e^{-3}	0.600	1.4
	hTNF143-145	7.76e^6	1.37e^{-3}	0.176	1.2
TPV-2LΔE328	hTNF	9.01e^5	7.18e^{-5}	0.08	1.8
	hTNF32-86	5.86e^6	5.02e^{-3}	0.857	1.6
	hTNF143-145	1.92e^5	1.22e^{-4}	0.637	1.9
TPV-2LΔC311	hTNF	1.56e^6	1.67e^{-4}	0.106	2.0
	hTNF32-86	2.32e^6	9.22e^{-3}	4.0	1.4
	hTNF143-145	6.6e^5	5.99e^{-4}	0.907	1.9

Association (*Ka*) and dissociation (*Kd*) rates as well as affinity constants (K_D) of TPV-2L mutants for the hTNF and its mutants as determined by SPR are indicated.

2LΔC311, the binding affinity for hTNF32-86 was further reduced by about 10-fold compared to the native TPV-2L (compare Figs. 3A and C and Table 1). This reduction is partly because of slower association and faster dissociation rate with TPV-2LΔC311 and further support the interaction of mutant residues of TNF with C-terminus of TPV-2L. However, deletion of C-terminal residues from TPV-2L had only modest reduction in binding affinity for hTNF143–145. C-terminal deletion of 42 amino acids of TPV-2L protein completely abolished the binding of both native and mutant form of TNF with TPV-2L.

Inhibition of human TNF mediated cytotoxicity by TPV-2L mutants

The inhibition of TNF-mediated biological activity by TPV-2L deletion proteins was tested with a standard cytotoxicity bioassay using murine L929 cells (Rahman et al., 2006). Sequential deletion of C-terminal residues from TPV-2L also reduced the inhibitory activity against hTNF gradually (Fig. 4). The IC_{50} of native TPV-2L to neutralize hTNF is less than 1 nM but in case of TPV-2LΔE328 and TPV-2LΔC311 it increased to 4 and 8 nM, respectively. However, larger deletions of TPV-2L lacked the ability to inhibit hTNF cytotoxity, in agreement with the hTNF binding experiments. None of the internal deletions of TPV-2L prevented hTNF mediated cytotoxity (data not shown).

Interaction of TPV-2L with human β2-microglobulin

Class I MHC molecules are polymorphic glycoproteins composed of a membrane bound variable heavy chain associated with a non-polymorphic light chain, β2 microglobulin. In virus-infected cells, MHC heterodimeric molecules bind peptides derived from viral proteins, which T cells recognize via their T-cell receptor chains (Townsend and Bodmer, 1989). To counteract this, many viruses have evolved mechanisms to regulate the function of class I MHC molecules. For example, mouse and human cytomegaloviruses exploit virus-encoded MHC class I homolog to protect the virus-infected cells from killing. Like the cellular class I MHC heavy chain molecules, the viral proteins also associate with β2m (Chapman and Bjorkman, 1998; Farrell et al., 1997). A human poxvirus, Molluscum contagiosum virus (MCV), also encodes an MHC class I homolog (MC80R) and can form complexes with β2m (Senkevich and Moss, 1998). Although TPV-2L has relatively less sequence similarity with cellular MHC class I molecule than the above examples, it has almost no similarity with any other known viral orthologs of MHC class I molecules. To examine whether this apparent difference is functionally significant, we have tested the ability of TPV-2L protein to interact with hβ2m. TPV-2L and the deletion proteins were co-expressed with hβ2m using *in vitro* TnT expression system (expressed from the T7 promoter present in the

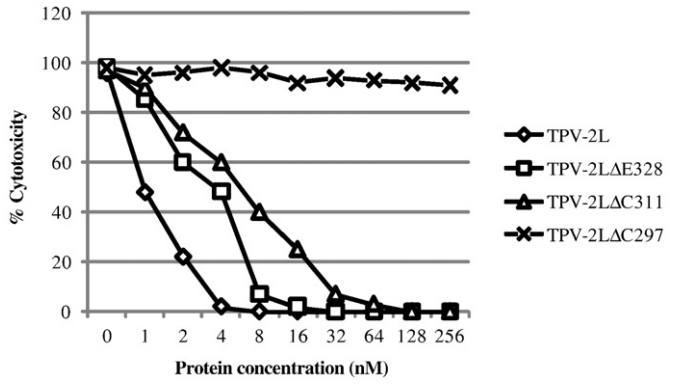

Fig. 4. Inhibition of TNF-mediated cellular cytotoxicity by TPV-2L mutants. Crystal violet staining was used to determine the percentage murine L929 cell viability after 12 h of treatment with hTNF and increasing concentration of TPV-2L deletion proteins. The values represent the median of three independent tests.

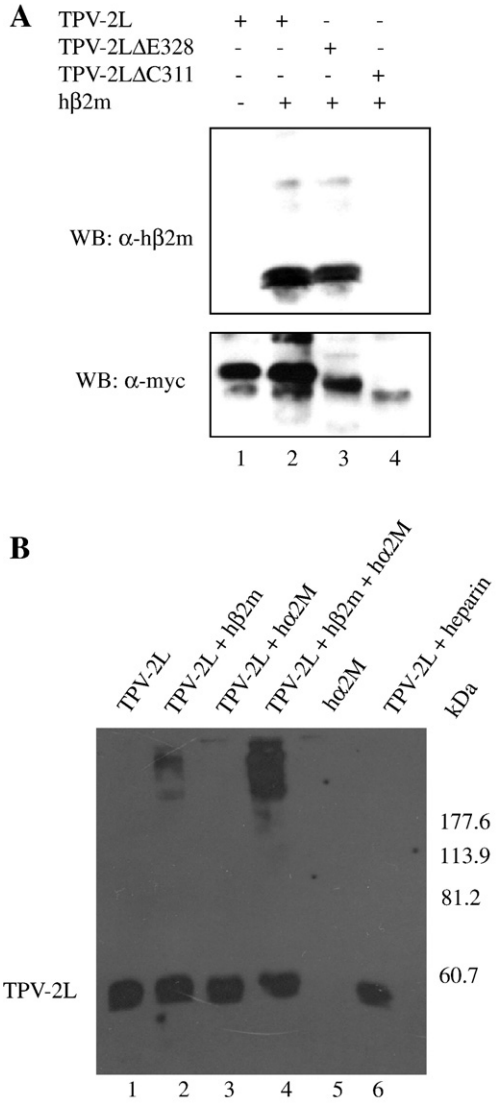

Fig. 5. Interaction of TPV-2L with human β2-microglobulin. (A) TPV-2L deletion proteins were co-expressed with hβ2m using TnT system. The reaction was incubated at 37 °C for additional 1 h and precipitated with Ni^{2+} beads. The samples were analyzed by SDS-PAGE and Western blot for the presence of co-precipitating protein. (B) TPV-2L, hβ2m and hα2M were co-incubated at 37 °C for 1 h, separated using non-reducing SDS-PAGE and visualized by Western Blot. Anti-myc antibody in the dilution of 1:5000 in 5% milk in TBS was used, followed by 1:5000 anti-mouse conjugated with HRP in 5% milk in TBS. Molecular size markers are indicated in kDa.

plasmid) and the complex was pulled down using Ni^{2+} chelate resin. Since TPV-2L and the deletions all have His-tag at the C terminus, they bound to the resin and the complex was detected using specific antibody. Co-expression of TPV-2L and hβ2m resulted in the pull down of hβ2m from the complex, as detected using anti-hβ2m antibody (Fig. 5A, lanes 2 and 3). We also assessed the ability of TPV-2L deletions to interact with hβ2m. TPV-2LΔE328 still has the ability to interact with hβ2m but not in the case of TPV-2LΔC311 (Fig. 5A, lanes 3 and 4). This suggests that unlike human TNF the amino acid residues located between E328 and C311 are important for binding with hβ2m. From this deletion studies it is also clear that the mechanism of interaction for TNF and hβ2m with TPV-2L could be different.

In order to confirm the complex formation between TPV-2L and hβ2m a gel-shift experiment was performed (Fig. 5B). TPV-2L protein alone, or together with hβ2m with or without human alpha 2 macroglobulin (hα2M), are co-incubated at 37 °C for 1 h. TPV-2L alone produced no shifted complex but in contrast, a high molecular weight complex appeared when TPV-2L and hβ2m were co-incubated indicating binding of the two proteins into a larger complex (Fig. 5B, lane b). The presence of hβ2m in the higher molecular mass was also confirmed with antibody against hβ2m (data not shown). When TPV-2L was co-incubated with hα2M, there was no interaction (Fig. 5B, lane c) but, interestingly, when TPV-2L, hβ2m and hα2M (lane d) were combined, a distinct higher molecular mass species was detected. Again, the presence of hα2M in this higher molecular mass was confirmed with antibody against hα2M (data not shown). However, the gel conditions do not reflect the actual sizes of the complexes (Fig. 5B, lanes b and d). As a control, hα2M alone or TPV-2L co-incubated with heparin did not show any obvious shifted complex in the gel. This result suggests that TPV-2L alone can interact with hβ2m but not with hα2M, as indicated by the shift to a higher molecular mass. However, when TPV-2L is bound with hβ2m, then this complex is now able to interact with hα2M. Further suggesting that the ordered sequence of protein binding (TPV-2L-hβ2m first, and then hα2M) is important for the interaction of TPV-2L. It has been reported that β2m has the capacity to bind to α2M, a serum anti-protease (Gouin-Charnet et al., 2000). There is further evidence that α2M with some conformational change, acquires the ability to bind a variety of cytokines such as IL-2, IFN-γ and TNF-α (James et al., 1992; Legres et al., 1995). TPV may gain additional anti-immune arsenal from such interactions.

Materials and methods

Reagents and cells

Recombinant human TNF, human IL-2, human IL-5, and human β2 microglobulin, were obtained from R and D biosystems, human α2 macroglobulin was obtained from Fitzgerald Industries International Inc. Human TNF mutants hTNFR32W-S86T and hTNFD143N-A145R were produced as described before (Rahman et al., 2006). Sf21 cells (Invitrogen) were cultured in SF-900 II serum-free medium (Invitrogen) and grown at 27 °C. Mouse L929 cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum.

Generation of TPV-2L deletion constructs for protein expression in the baculovirus system

The TPV-2L deletion constructs were made using polymerase chain reaction (PCR) and oligonucleotides. The plasmid, pcDNA-TPV-2L containing the complete TPV-2L open reading frame was used as template (Brunetti et al., 2003). The desired deleted sequences were PCR amplified with specific oligonucleotides (Supplementary Table S1) and Pfu DNA polymerase (Stratagene) using TPV-2L as template. PCR amplified DNA was digested with EcoRI and XhoI and cloned into pcDNA3.1/Myc/His (Invitrogen) plasmid in order to make C-terminal Myc and His_6 fusion. The correct sequence of all the cloned DNA fragments was confirmed by DNA sequencing.

Internal deletions of TPV-2L were made by amplification of two fragments lacking the deleted region and the two fragments were annealed and reamplified using the N-terminal and C-terminal primers of the gene. PCR was used to amplify the upstream and downstream regions of the desired deletion sequences with specific oligonucleotides and pfu DNA polymerase using TPV-2L sequence. The primer pairs for internal deletions are presented in Supplementary Table S1. Products from the two PCR reactions were purified using agarose gel electrophoresis and the two products with overlapping termini were mixed, denatured by heating to 95 °C and allowed to re-anneal. DNA polymerase and dNTPs were added for 3′ extension of the overlapping termini, followed by a second round of PCR amplification of the full length extension using TPV-2L forward and reverse primers. The products were digested with EcoRI and XhoI and cloned in pcDNA3.1/Myc/His to make C-terminal Myc and His_6 fusion. Using the same method all other deletion constructs were made. All clones were sequenced to ensure that mutations had not been introduced during the amplification. The deleted clones were further transferred in pFastBac1 (Invitrogen) plasmid.

Generation of recombinant baculoviruses

The TPV-2L deletions and truncations in pcDNA3.1 plasmid were transferred into pFastBac1 plasmid (Invitrogen) and recombinant baculoviruses were produced using the Bac-to-Bac expression system following the manufacturers protocols (Invitrogen). Briefly, the plasmids were transformed into competent DH10Bac bacteria, where a transposition event generated the corresponding recombinant bacmids. The purified bacmid DNA were transfected into Sf21 insect cells and the recombinant baculoviruses were harvested from the cell culture supernatants 3–5 days after transfection. These viruses were further amplified in one step to generate a higher titer recombinant virus stock for protein production.

Protein purification

For protein expression Sf21 cells were infected with recombinant baculoviruses at high MOI of 5–10 pfu/cell. Cell supernatants were harvested at 3–4 days postinfection (pi), clarified by centrifugation at 2000 ×*g* for 10 min followed by high speed at 30,000 ×*g* for 30 min and then concentrated 10-fold using protein concentrator (Pall Life Sciences). The concentrated supernatants were then buffer-exchanged against phosphate buffer, pH 7.0, containing 10 mM imidazole. Protein was purified by metal chelate affinity chromatography (Ni^{2+}/Co^{2+}) following the manufacturer's protocol (Invitrogen). Purified protein was analyzed by 10% acrylamide SDS-PAGE stained with Coomassie Blue R250. The protein concentration was measured by the Bradford assay or absorbance measurements at 280 nm.

Cytolytic assays

Mouse L929 cells were used to test TNF-mediated cytotoxicity using a crystal violet staining method (Rahman et al., 2006). In this method 10^5 cells per well were seeded in 12-well plates in a total volume of 500 μl of cell growth medium (DMEM with 10% FBS) and incubated overnight at 37 °C. The next day the medium was removed and cells were treated with medium containing 5 μg/ml actinomycin D and 1 ng/ml human TNF in the presence or absence of serial dilutions of TPV-2L proteins. The cells were incubated for 18 h at 37 °C,

washed three times with PBS and stained for 10 min with 200 μl of 0.5% crystal violet in water. Plates were washed with water and the dye was solubilized with 2% sodium deoxycholate and the absorbance (A) was determined at 570 nm. All assays were performed in triplicate. Percentage of cytolysis was quantified by following equation:

$$\%C = \left[A_{(\text{control well})} - A_{(\text{test})}\right] / \left[A_{(\text{control well})}\right] \times 100.$$

Analysis of TNF binding specificity and affinity constants

TNF binding specificity and affinity constants were estimated by Surface Plasmon Resonance (SPR) using a Biacore X biosensor (Biacore, GE Healthcare). Screening of TNF binding was done using the NTA sensor chip (Biacore) as described before (Rahman et al., 2006). In brief, the sensor chip surface was activated with 500 μM $NiCl_2$ solution in eluent buffer followed by immobilization of the protein by injection of 50 nM His-tagged protein at a flow rate of 2–5 μl/min until the RU (Response Difference) reached >3000. To monitor the binding, human TNF was injected at a rate of 30 μl/min for 3 min. The sensor chip surface was regenerated by stripping nickel from the surface by injection of regeneration solution containing EDTA.

Kinetic analysis of the recombinant TPV-2L proteins was done by standard amine coupling chemistry using CM5 chips. The proteins were immobilized at low densities of 500 RU and different concentrations of hTNF were injected at a flow rate of 50 μl/min over a period of 2 min and allowed to dissociate for an additional 5 min by allowing the buffer to flow. The surface was regenerated after each injection using 10 mM acetate pH 4.0 or 10 mM glycine–HCl pH 1.5. The data were analyzed globally with the BIAevaluation 3.0 software by using a 1:1 Langmuir model.

Immunoblotting

Protein samples were separated on SDS-PAGE gels and transferred to PVDF membrane (GE Healthcare) using a semidry transfer apparatus (Biorad). Membranes were blocked in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween-20 pH 7.6) containing 5% non-fat dry milk for 1 h at room temperature and then incubated with primary antibodies mouse anti-myc (Santa Cruz), mouse anti-h β2m (Santa Cruz) overnight at 4 °C. The membranes were washed three times for 10 min each with TBST and incubated with goat-anti-mouse-HRP as secondary antibody in TBST containing 5% non-fat dry milk for 1 h at room temperature with gentle agitation. The membrane was washed three times for 10 min each with TBST, and the signal was detected by applying chemiluminescence substrate (Pierce) and exposed to x-ray film (Eastman Kodak).

In vitro transcription/translation of plasmid constructs and pull-down experiments

The rabbit reticulocyte coupled transcription and translation (TnT) system (Promega) was used according to manufacturer's protocol for expression of proteins in vitro. Protein expression was confirmed by running 2.5 μl of the TnT reaction on SDS-PAGE gels and western blot analysis using anti-myc antibody (Santa cruz) for the Myc-His tagged protein and specific antibody for the untagged proteins. For pull-down the two plasmids were co-expressed using TnT in 10 μl reaction. 2 μl of the reaction was used for confirming protein expression by Western blot analysis. Remaining of the reaction was incubated at 37 °C water bath for 1 h for the formation of protein complex. For pull-down, Ni^{2+} beads (20 μl) were equilibrated in binding buffer (50 mM NaH_2PO_4, pH 8.0, 500 mM NaCl and 20 mM imidazole) and added to the protein complex followed by incubation for 4 h at 4 °C. The beads were collected by centrifugation (60 s, 3000 rpm), the supernatant was discharged and the beads were subsequently washed there times with 300 μl of binding buffer. The bound proteins were eluted from the beads with SDS-PAGE sample buffer and analyzed by SDS-PAGE and Western blot analysis.

TPV-2L co-incubation experiment

The TPV-2L protein containing baculovirus supernatant (5 μL) or purified TPV-2L protein (1 μg/μl) was incubated with 1 μL of human-β-2 microglobulin (1 μg/μL in PBS) or 1 μL of human-α-2 macroglobulin (1 μg/μL in PBS), alone or in combination of both the proteins in a volume of 10 μl for 60 min in a 37°C water bath. Sterile PBS was added to achieve consistent sample volume prior to the addition of equal volume of SDS-gel loading buffer (20% Glycerine, 4.6% SDS, 0.002% bromophenol blue) without reducing agent. A sample of 3 μL of sodium heparin (1 μg/μL in PBS, Sigma), mixed with TPV-2L protein was used as a control. Samples were separated on a 10% Tris–Glycine gel at a constant 100 V for 2 h 30 min and detected by Western blot analysis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.01.026.

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