Characterizing Functional Domains for TIM-Mediated Enveloped Virus Entry

Sven Moller-Tank, Lorraine M. Albritton, Paul D. Rennert, Wendy Maury

Department of Microbiology, University of Iowa, Iowa City, Iowa, USA; Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee, USA; SugarCone Biotech LLC, Holliston, Massachusetts, USA

ABSTRACT

T-cell immunoglobulin and mucin domain 1 (TIM-1) and other TIM family members were recently identified as phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors (PVEERs). These proteins enhance entry of Ebola virus (EBOV) and other viruses by binding PtdSer on the viral envelope, concentrating virus on the cell surface, and promoting subsequent internalization. The PtdSer-binding activity of the immunoglobulin-like variable (IgV) domain is essential for both virus binding and internalization by TIM-1. However, TIM-3, whose IgV domain also binds PtdSer, does not effectively enhance virus entry, indicating that other domains of TIM proteins are functionally important. Here, we investigate the domains supporting enhancement of enveloped virus entry, thereby defining the features necessary for a functional PVEER. Using a variety of chimeras and deletion mutants, we found that in addition to a functional PtdSer-binding domain PVEERs require a stalk domain of sufficient length, containing sequences that promote an extended structure. Neither the cytoplasmic nor the transmembrane domain of TIM-1 is essential for enhancing virus entry, provided the protein is still plasma membrane bound. Based on these defined characteristics, we generated a mimic lacking TIM sequences and composed of annexin V, the mucin-like domain of α-dystroglycan, and a glycosphatidylinositol anchor that functioned as a PVEER to enhance transduction of virions displaying Ebola, Chikungunya, Ross River, or Sindbis virus glycoproteins. This identification of the key features necessary for PtdSer-mediated enhancement of virus entry provides a basis for more effective recognition of unknown PVEERs.

IMPORTANCE

T-cell immunoglobulin and mucin domain 1 (TIM-1) and other TIM family members are recently identified phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors (PVEERs). These proteins enhance virus entry by binding the phospholipid, PtdSer, present on the viral membrane. While it is known that the PtdSer binding is essential for the PVEER function of TIM-1, TIM-3 shares this binding activity but does not enhance virus entry. No comprehensive studies have been done to characterize the other domains of TIM-1. In this study, using a variety of chimeric proteins and deletion mutants, we define the features necessary for a functional PVEER. With these features in mind, we generated a TIM-1 mimic using functionally similar domains from other proteins. This mimic, like TIM-1, effectively enhanced transduction. These studies provide insight into the key features necessary for PVEERs and will allow for more effective identification of unknown PVEERs.

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Address correspondence to Wendy Maury, wendy-maury@uiowa.edu.
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enveloped viruses that do not have glycoproteins (2, 5). There are two likely mechanisms of entry enhancement that are not mutually exclusive; either TIMs directly internalize virions into endosomes or TIM/virion complexes interact with additional PtdSer-binding proteins that facilitate internalization.

In this study, we define the necessary features of the TIM family of proteins for the enhancement of Ebola virus (EBOV) entry. In addition to conserved PtdSer-binding elements of the IgV domain, we found that the length and the domain structure of the MLD are critical for virus binding and transduction. However, the presence of the transmembrane domain and cytoplasmic tail is nonessential for virus entry as a glycosylphosphatidylinositol (GPI) anchor can replace these elements, demonstrating that TIM cytoplasmic tail signaling is not critical for enhancing EBOV transduction. With knowledge gained from the identification of domains that are required for virus entry, we built a chimeric molecule composed of a GPI anchor, the stalk of α-dystroglycan, and annexin V that enhanced EBOV entry as efficiently as wild-type (WT) TIM-1.

MATERIALS AND METHODS

Cell lines and viruses. HEK 293T cells, a human embryonic kidney cell line were maintained in Dulbecco modified Eagle medium (Gibco-BRL) with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

Vesicular stomatitis virus (VSV) pseudovirions were produced as previously described (4, 14–16). We used VSV (strain Indiana) pseudovirions whose genomes had the G glycoprotein gene replaced with enhanced green fluorescent protein (VSVΔG-EGFP). To produce VSV pseudovirions, HEK 293T cells were transfected with plasmids expressing either EBOV GP lacking the mucin domain of GP1, Lassa virus (LASV) glycoprotein precursor (GPC), Sindbis virus (SINV) 2.2 1LIL envelope protein (env), Ross River virus (RRV) GP, GP64, or Chikungunya virus (CHIKV) env (strain OPY1) and transduced 24 h later with VSVΔG-EGFP pseudovirions. After 4 h of virus uptake, the plates were washed and refreshed with media. Pseudotyped virions were collected in supernatant 48 and 72 h after transduction, pooled, and filtered through a 0.45-μm-pore-size filter. Virus aliquots were stored at −80°C until use.

Fluorescein isothiocyanate (FITC)-labeled EBOV GP pseudovirions were generated as previously described (5). Briefly, pseudovirions were concentrated by centrifuging supernatants overnight at 4°C at 5,380 × g, resuspended in 500 mM carbonate buffer (pH 9.5), and reacted with FITC-Isomer 1 (Invitrogen) at 4°C for 1 h in the dark. Pseudovirions were then dialyzed into 1X phosphate-buffered saline (PBS) using a 10,000-molecular-weight-cutoff Slide-A-Lyzer dialysis cassettes (Thermo Scientific) and purified by ultracentrifugation through a 20% sucrose cushion at 80,000 × g at 4°C. Pellets were resuspended in 1X PBS, filtered through a 0.45-μm-pore-size syringe filter, divided into aliquots, and stored at −80°C until use.

Transductions. HEK 293T cells were transfected with plasmids as indicated in figures using polyethyleneimine according to a standard protocol (17). At 24 h after transfection, one portion of the cell population was transferred to a six-well tissue culture plate and stained for surface expression 24 h later as described below. A second portion of the transfected cells was transferred to a 48-well tissue culture plate and transduced 24 h later with pseudovirions at the multiplicities of infection (MOIs) noted in the figure legends. At 24 h after transduction, the cells were detached with Accutase solution (Millipore), and EGFP expression was assessed by flow cytometry as the percent positive cells in the FL-1 channel. Transduction data were normalized as indicated in the figure legends.

For the transduction into cells expressing mixtures of MLD mutants, the DNA for MLD mutant expression vectors were mixed together in equal ratios of DNA in the following combinations: (C66 alone, C66+C84, C66+C84+C101, C84 alone, C84+C101, C84+C101+C118, C101 alone, C101+C118, and C66+C84+C101+C118). All cell populations were transfected with the same total mass of DNA; thus, the mass of DNA used for one mutant expression vector in a mixture of three mutants would be one-third the mass of DNA used in the transfection of that mutant alone.

Surface expression. Transfected HEK 293T cells were detached with 1X PBS plus 5 mM EDTA 48 h after transfection, washed with 1X PBS plus 5% FBS, and incubated for 1 h with goat polyclonal antisera specific to mTIM-1, TIM-1, TIM-3, or TIM-4 as indicated (R&D Systems) at a concentration of 4 μg/ml. Alternatively, cells were incubated for 1 h with human TIM-1 specific mouse monoclonal antibodies (MAbs) ARD5 or AKG7 (4, 18, 19) as indicated at a concentration of 5 μg/ml. Normal goat serum or purified mouse IgG2a (R&D Systems) were used as negative controls. Cells were washed and incubated for 20 min with anti-mouse or anti-goat secondary antisera conjugated to either Cy5 (Invitrogen) or DyLight 649 (Jackson ImmunoResearch). After incubation, cells were washed and protein expression was assessed by measuring the percentage of positive cells in the FL-4 channel using a FACSCalibur flow cytometer (BD Biosciences). All flow cytometry data were analyzed using FlowJo software (TreeStar, Inc.).

Generation of TIM mutants and chimeras. TIM-1 (NM_012106.2), TIM-3 (NM_032782.4), TIM-4 (NM_133879.2), and mTIM-1 (NM_134248.2) cDNAs were cloned downstream and expressed from the human cytomegalovirus (CMV) immediate-early promoter in pCMV6-XL5. All wild-type and chimeric constructs were sequenced in their entirety to verify the correct composition.

(i) TIM IgV chimeras. Chimeras were generated by PCR amplification of the IgV domains with overhanging primers containing restriction enzyme sites. Subsequently, both vector and PCR product were digested with respective enzyme pairs, gel purified, and ligated together. In all cases, the restriction sites excised the IgV domain from the parent vector. The sequences encoding the IgV domains of mTIM-1 or TIM-3 were cloned into a TIM-1 expression plasmid using 5′ KpnI and 3′ MfeI restriction sites to create chimeras m1h1 or h1h3 and cloned into a TIM-4 expression plasmid using 5′ EcoRI and 3′ BamHI restriction sites to create h1h4 or h3h4, respectively. Initially a BamHI restriction enzyme site was inserted downstream of the TIM-4 IgV domain, introducing an A134G missense mutation that had no effect on EBOV transduction (data not shown). The sequences encoding the IgV domain of TIM-1 were cloned into a TIM-3 expression plasmid using 5′ NheI and 3′ BstEII restriction sites to create h1h3.

(ii) TIM-1 MLD length mutants. Primers pairs containing overhanging 5′ MfeI and 3′ PstI restriction enzyme sites were designed to amplify various regions between the MfeI and PstI restriction sites present within the TIM-1 MLD coding region of the expression plasmid. PCR products and TIM-1 expression vector were digested with MfeI and PstI, separated on an agarose gel, purified, and ligated. Mutations were confirmed by sequencing. The sequences are aligned in Fig. 2A.

(iii) TIM-1 cytoplasmic tail mutants. The TIM-1 construct lacking the cytoplasmic tail domain (h1Δcyto) has been previously described (18). The TIM-3, TIM-4, and mTIM-1 cytoplasmic tail deletions (h3Δcyto, h4Δcyto, and m1Δcyto) were created by mutating Y227, L340, or S268, respectively, to stop codons, resulting in early termination after the transmembrane domain, while still retaining a few cytoplasmic domain, charged residues.

The TIM-1 cytoplasmic tail exchange mutants were created by first introducing a BamHI site downstream of the transmembrane domain, resulting in a YF320GS mutation that had no effect on expression or EBOV transduction (data not shown). The sequences encoding the cytoplasmic tails of TIM-3, TIM-4, and mTIM-1 were amplified by PCR with primers encoding overhanging BamHI (TIM-3 and TIM-4) or BglII (mTIM-1) and Xbal restriction sites. After excision of the TIM-1 cytoplasmic tail using BamHI and Xbal restriction sites, these PCR products were ligated into the modified TIM-1 plasmid to construct TIM-1 chimeras containing the cytoplasmic tail of either mTIM-1 (h1m1cyto), TIM-3 (h1h3cyto), or TIM-4 (h1h4cyto).
(iv) TIM-1 GPI-anchored mutants. A plasmid expressing GPI-anchored murine TIM-4 (mTIM-4) in which the transmembrane and cytoplasmic tail domains of mTIM-4 are replaced with the carboxy-terminal 37 amino acids (aa) of decay-accelerating factor (DAF) was kindly provided by Koki Ravichandran (University of Virginia) (20). The sequences encoding the IgV and MLD domains of TIM-1, TIM-3, TIM-4, and mTIM-1 were amplified by PCR using primers encoding overhanging 5’ BglII and 3’ SbfI restriction enzyme sites and ligated into the GPI-anchored mTIM-4 plasmid after digestion of the vector at these same sites. This resulted in GPI-anchored mTIM-1 (m1-GPI), TIM-3 (h3-GPI), and TIM-4 (h4-GPI) expression plasmids.

A protocol used by Park et al. (20) was modified to confirm GPI linkage of our mutants. Briefly, HEK 293T cells transfected with wild-type (WT) TIMs or GPI-anchored TIM expression constructs were plated in 24-well plates and incubated 48 h later with or without 0.4 U of phosphatidylinositol-specific phospholipase C (PI-PLC, Invitrogen) at 37°C for 1 h in 100 μl of PBS. PI-PLC cleaves the phosphoglycerol bond within GPI, releasing GPI-anchored proteins from the membrane. Flow cytometry analysis of surface-stained cells confirmed that the expression of GPI-anchored TIMs was reduced from cell surface after PI-PLC treatment, whereas there was no detectable loss of WT TIMs (see Fig. 1H).

(v) αDG chimeras. The MLD of TIM-1 was replaced with that of murine α-dystroglycan (αDG) to generate αDG-MLD. The cDNA of murine dystroglycan (DAG1) was kindly provided by Kevin Campbell (University of Iowa). The sequences encoding the MLD of murine αDG (aa 315 to 463) and the transmembrane and cytoplasmic domains of human TIM-1 (aa 295 to 364) were amplified by PCR, fused by an additional PCR amplification using an overlapping primer set, and cloned into an expression vector containing the signal peptide and IgV domain sequences of TIM-1. The percent identity between MLD of αDG (aa 314 to 483) and TIM-1 (aa 128 to 294) was determined by CLUSTAL W alignment of amino acid sequences using MegAlign software (DNASTAR).

A construct containing both the MLDs of TIM-1 and αDG, called 2×MLD, was generated by inserting the TIM-1 IgV and αDG MLD sequences from αDG-MLD upstream of the MLD and cytoplasmic domains of TIM-1. Thereby, the MLD of αDG is between the IgV and MLD of TIM-1.

(vi) AnxV-αDG-GPI chimeras. A plasmid encoding human annexin V (AnxV) was kindly provided by Seamus Martin (Trinity College, Dublin, Ireland). The sequences encoding AnxV and the MLD of murine αDG (aa 314 to 483) were amplified by PCR, joined by a second PCR amplification using primers with overhanging BglII and SbfI restriction sites, and ligated into the mTIM-4 GPI vector between the immunoglobulin κ-chain leader sequence and the DAF sequence, replacing the mTIM-4 coding sequence.

(vii) PRR chimeras. h1PRR was generated using In-Fusion HD (Clontech) following the manufacturers protocol. Two fragments encoding aa 242 to 300 and 239 to 321 of the proline-rich region (PRR) from the amphotrophic 4070A murine leukemia virus (MuLV) envelope gene, kindly provided by Paul McIlroy (University of Iowa), were amplified by PCR. These fragments were inserted in tandem between sequences encoding aa 129 and 295 of WT TIM-1 to create a chimera in which the MLD of TIM-1 is replaced with two copies of the PRR from MuLV env.

(viii) RAGE chimeras. h1RAGE was generated by first PCR amplifying the IgV domain of TIM-1 (aa 1 to 125) and sequences downstream of the human receptor for advanced glycation end products (RAGE) IgV (aa 119 to 338) (Origene). These amino acid residues of RAGE compose the two IgC2, transmembrane, and cytoplasmic domains of the protein. These TIM-1 and RAGE sequences were joined by a second PCR amplification and ligated into a vector, downstream of the CMV promoter.

h1RAGEh1cyto was generated using In-Fusion HD. The h1RAGE vector and ectodomain of RAGE were amplified by PCR and joined according to the manufacturer’s protocol. The resulting construct contained the IgV domain of TIM-1 (aa 1 to 124), the extracellular domains of RAGE without the RAGE IgV domain (aa 119 to 338), and the transmembrane and cytoplasmic domains of TIM-1 (aa 295 to 364).

h1RAGEmucin was constructed similarly to h1RAGE, resulting in a portion of the TIM-1 MLD and entire transmembrane and cytoplasmic domains of TIM-1 (aa 209 to 364) being inserted downstream of the RAGE stalk sequence (aa 119 to 335) of h1RAGE.

FITC-pseudovirion binding assay. At 48 h after transfection with TIM-1, empty vector, or MLD TIM-1 mutants, HEK 293T cells were incubated with FITC-labeled EBOV GP pseudovirions (MOI of 2.25 as determined from titers on Vero cells) for 1 h on ice. The cells were washed three times with 1× PBS plus 5% FBS, and the cellular fluorescence was either determined immediately by flow cytometry or the cells were additionally stained with AKG7 as described above prior to flow cytometry for analysis of TIM-1 cell surface expression.

Enzyme-linked immunosorbent assays (ELISAs). Soluble TIM-1 was produced in HEK 293T cells by transfecting in an expression vector, previously described (5), in which the transmembrane and cytoplasmic domains of TIM-1 are replaced with a hemagglutinin (HA) tag. Supernatant was collected 48 h after transfection. TIM-1 MLD mutants were similarly generated after subcloning of the mutants described above into the soluble WT TIM-1 expression vector. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Western blot), or serial dilutions of proteins were applied directly to membranes and pulled through via vacuum by using a dot blot apparatus (dot blot). Nitrocellulose was blocked for 2 h in 1× PBS with 10% nonfat dry milk and subsequently incubated for 2 h with anti-HA antibody (1:4,000) in 1× PBS with 10% milk and 0.15% Tween 20. Protein expression was imaged using a LI-CORE Odyssey CLx and quantified by measuring the total fluorescent signal and subtracting background signal using Image Studio (LI-CORE).

The protein levels were normalized by first plotting fluorescent data from dot blots against the dilution factor for each mutant TIM-1 and WT TIM-1. A line was fitted to the scatter plot for each construct, and data points outside the linear range due to saturation of the nitrocellulose were eliminated. The slope of these lines was then used to normalize relative amounts of supernatant to use for each mutant and WT TIM-1. Appropriate normalization of dilutions was confirmed by Western blotting.

Nunc MaxiSorp ELISA plates were precoated overnight with PtdSer liposomes (50 μM) diluted in 1× TBS+ (150 mM NaCl, 25 mM Tris, 10 mM CaCl2 [pH 7.2]). PtdSer liposomes were generated as previously described (5). Supernatants were diluted in 1× TBS+ to normalize TIM-1 protein amounts as described above. Plates were blocked for 2 h at 4°C with 1× TBS+ with 2% bovine serum albumin, incubated with serial dilutions of supernatants for 2 h, probed with rabbit polyclonal anti-HA antiserum (Sigma) for 1 h and horseradish peroxidase-conjugated secondary anti-rabbit antibody for 1 h, and developed using 1-Step Ultra TMB-ELISA (Thermo). Plates were washed thoroughly between steps with 1× TBS+. The absorbance was read at 450 nm.

RESULTS
TIM family members enhance EBOV entry. We and others recently identified TIM-1 and TIM-4 as PVEERs (2–5). The IgV domains of TIM family members share a conserved PtdSer-binding pocket responsible for interacting with PtdSer on the virion surface and essential for TIM-1-mediated enhancement of virus entry. However, despite containing a PtdSer-binding pocket, TIM-3 does not effectively function as a PVEER (2–4). In these studies, we sought to determine the structural basis for the high PVEER efficiency of TIM-1 compared to TIM-3. In addition, we wanted to test whether the murine homolog of human TIM-1 (mTIM-1) that shares many of the same structural features also mediates enveloped virus transduction.

PVEER efficiency was quantified by measuring transduction of pseudotyped vesicular stomatitis virus (VSV) that expressed EGFP as reporter molecule in place of native G. Although TIM-1
enhances the entry of a number of enveloped viruses, EBOV GP pseudotyped VSV (EBOV pseudovirions) entry is most strongly enhanced by TIM-1, whereas the entry of LASV GPC pseudotyped VSV (LASV pseudovirions) entry is unaffected by TIM-1 expression (2, 4, 5). Thus, stocks of these two pseudovirions were used to identify TIM sequences required for virus transduction. EBOV GP lacking the GP1 mucin domain was used since this protein confers the same tropism as the full-length Ebola virus GP and produces higher pseudovirus titers (15, 21, 22). However, entry mediated by the full-length Ebola virus GP is enhanced in an equivalent manner to the mucin domain deleted GP by the expression of TIM-1 (2, 4, 5).

HEK 293T cells transiently expressing TIM-1, mTIM-1, TIM-3, or TIM-4 at comparable and robust levels on the cell surface (Fig. 1A) were transduced with EBOV and LASV pseudovirions. For comparison, cells transfected with empty vector were also transduced. Similar to TIM-1 and TIM-4, mTIM-1 increased EBOV transduction, but TIM-3 only slightly enhanced transduction (<2-fold) (Fig. 1B). Consistent with previous findings (2, 4, 5), TIM proteins did not enhance LASV entry, Thus, LASV pseudovirion entry was used throughout these studies as a control.

The IgV domains from all tested TIMs mediate entry. The ability of other TIMs to enhance EBOV entry more effectively than TIM-3 might be due to unique aspects of their IgV domains or...
higher affinity for PtdSer (12). This was tested by replacing the IgV domain of TIM-1 or TIM-4 with that of mTIM-1 (m1h1 and m1h4) or TIM-3 (h3h1 and h3h4). In addition, the IgV domain of TIM-3 was replaced with that of TIM-1 (h1h3). All chimeric TIM proteins were expressed abundantly on the surfaces of transfected HEK 293T cells (Fig. 1C). The chimeras containing the IgV domain of each TIM-1 or TIM-3 increased transduction of EBOV pseudovirions similar to WT TIM-1 (Fig. 1D). However, the h1h3 chimera containing the TIM-1 IgV domain and the remaining domains from TIM-3 was as deficient as WT TIM-3. These results indicated the IgV domain of TIM-3 is not responsible for its reduced efficacy to enhance EBOV transduction, but rather the other domains are.

**Cytoplasmic and transmembrane domains of TIM family are not essential for EBOV transduction.** Due to retention of the MLD, transmembrane domain, and cytoplasmic tail of TIM-4 or TIM-1 in our functional TIM-3 chimeras, we evaluated the contribution of these domains to transduction efficiency. TIM-1 (18), TIM-3 (23), and mTIM-1 (24, 25) have cytoplasmic tails that after tyrosine phosphorylation can potentiate cell signaling, which could contribute to transduction. However, the cytoplasmic tail of TIM-4 has no known signaling function (20). To determine the potential role of cytoplasmic domain signaling during EBOV transduction, we tested chimeras in which the cytoplasmic tail of TIM-1 was exchanged with that of mTIM-1 (h1m1cyto), TIM-3 (h1h3cyto), or TIM-4 (h1h4cyto). Transfection of chimeric TIM proteins resulted in equivalent transduction efficiencies (Fig. 1E), suggesting either no role for the cytoplasmic tail or a mechanism of signaling is shared between the cytoplasmic tails of TIM proteins.

To further investigate the importance of the cytoplasmic tail domains in EBOV transduction, we tested TIM mutants whose cytoplasmic tails were deleted. The TIM-3 and TIM-4 mutants (h3Δcyto and h4Δcyto) supported transduction in a manner similar to the WT proteins (Fig. 1F), while deletion of the TIM-1 or mTIM-1 cytoplasmic tails (h1Δcyto or m1Δcyto, respectively) resulted in an approximately 60 or 70% decrease. The decrease in transduction might be explained by reduced expression and, indeed, h1Δcyto and m1Δcyto were expressed on fewer cells compared to WT (Fig. 1G). In addition, the cytoplasmic or transmembrane domains of TIM-1 and mTIM-1 might contribute to appropriate localization of these proteins to plasma membrane. In order to examine this possibility, TIM family mutants were generated in which the ectodomains of each TIM composed of the IgV and MLD were anchored to the plasma membrane via a GPI anchor, as confirmed by PI-PLC treatment (Fig. 1H). Unlike the cytoplasmic deletion mutants, there were no significant differences in expression (Fig. 1H) or transduction (Fig. 1I) of any of the GPI-anchored TIMs compared to their WT counterparts. These results rule out a role for the transmembrane domain in transduction.

**Deletions in the TIM-1 MLD decrease virus binding and transduction.** Based on the results presented above, the differences in PVEER efficacy observed between TIM-3 and the other family members cannot be accounted for by the IgV, the transmembrane, or cytoplasmic domains, leading to a focus on the remaining domain of these proteins, the MLD. The MLDs of the TIM family members differ in several ways: amino acid sequence, extent of O- and N-linked glycosylation, and overall length. Noting that TIM-3 has a considerably shorter MLD (~66 aa) than TIM-1, mTIM-1, and TIM-4 (~167, 107, and 174 aa, respectively) and that significant deletion within the TIM-1 MLD inhibits virus uptake (2), we hypothesized that the length of the MLD might be important for PVEER function. For example, a minimal length of the MLD may be needed to project the IgV domain sufficiently far from the surface of the cell for interaction with virus. To test the role of the MLD length, a series of TIM-1 MLD mutants were generated by deleting various lengths within a highly repetitive region of the MLD. These mutants retained either amino-terminal (N), middle (M), or carboxy-terminal (C) sequences of this region, and the length of the MLD is noted in the name of each MLD mutant. The amino acid sequences of the TIM-1 MLD mutants are aligned with TIM-1 in Fig. 2A. In addition to deletions, some mutants had point mutations generated by introduction of the restriction sites.

Initial studies with this panel of mutants were performed to determine the effect that reductions of MLD length had on virus binding. We quantified FITC-labeled EBOV pseudovirion binding to cells transfected with WT TIM-1, a selection of MLD deletion mutants, or an empty vector. Mutants containing large MLD deletions such as C66 did not show appreciable increases in virion binding compared to cells expressing empty vector (Fig. 2B). However, as the MLD lengthened, virion binding increased, reaching levels comparable to the WT, suggesting that the length of the MLD is important for TIM-1 to bind EBOV.

To determine whether the cells expressing the largest amounts of these TIM constructs bound the greatest amounts of virus, we bound virus to MLD mutant-transfected HEK 293T cells and assessed the cells for both TIM-1 expression using the MLD-specific MAb AKG7 and binding of FITC-labeled virus. Except for C66, the MLD length mutants were detected at or near wild-type levels (Fig. 2C). An IgV domain MAb, ARD5, detected levels of surface-expressed C66 equivalent to those of the WT (data not shown), suggesting AKG7 detection of this mutant was suboptimal, perhaps due to steric hindrance by the IgV domain which is much closer to the AKG7 epitope in this mutant (26). As evidenced in the contour plots shown in Fig. 2C, virus binding was more robust on cells expressing higher levels of TIM-1. Quantification of binding from Fig. 2B illustrates a strong correlation between virus binding and MLD length (Fig. 2D). To determine whether TIM-1 MLD length is only important for binding virion-associated PtdSer in the context of the cell surface or whether soluble TIM-1 constructs with shortened MLDs also bound with reduced efficiency, we assessed the ability of various-length soluble TIM-1 mutants to bind PtdSer liposomes in an ELISA. Serial dilutions of normalized quantities of soluble HA-tagged MLD length mutant TIM proteins, as demonstrated by an immunoblot (Fig. 2E), were incubated with ELISA plates precoated with PtdSer. All MLD-length mutants bound to PtdSer liposomes similarly to the WT (Fig. 2F). We confirmed these studies with EBOV pseudovirions and found binding to be similar for soluble MLD mutants containing short or long MLDs (data not shown). Neither WT TIM-1 nor MLD mutants bound to plates alone nor plates coated with phosphatidylcholine (data not shown). Thus, while reduced virion binding of short MLD mutants was observed when the TIM was membrane associated, this was not observed with soluble mutant proteins.

To determine whether the reduction in virus binding correlated with reduced ability to enhance virus entry, we evaluated the transduction mediated by all 18 of the MLD deletion mutants. The enhancement of EBOV transduction correlated with increasing
MLD length, the effect plateauing at WT TIM-1 levels of transduction (Fig. 3A to C). A similar plateau did not occur in the binding studies, likely due to our ability to detect binding of multiple virions to the same cell by flow cytometry, but not transduction of multiple virions into a single target cell by an increase in EGFP expression. Notably, wild-type levels of PVEER function were seen regardless of if amino-, carboxy-terminal, or combinations of both deletions were present, provided the overall length of the MLD was ~120 residues or longer. A scatter plot of the binding data from Fig. 2D and the transduction data from Fig. 3A showed a positive correlation between ability of the TIM-1 mutants to bind to EBOV pseudovirions and enhance transduction (Fig. 3D).
sequences (Fig. 4A).

serines, and prolines, there is only 19.9% identity between the two MLD chimera. While both MLD sequences are rich in threonines, this

efficiency into mixed or single MLD mutants, WT, or empty vector the C66, C84, C101, and C118 MLD mutants. Transduction effi-
cients are normalized to TIM-1 expression as assessed by surface staining (% TIM-positive cells), and all data are means ± the SEM of at least three replicates. For panel A, significance was calculated by using a one-sample t test comparison to 100 (**, P < 0.001).

We hypothesized that expressing MLD mutants of different lengths on the same cell might have a synergistic effect due to mutants with longer MLDs passing virus off to those with shorter MLDs, thereby enhancing the binding capacity of the shorter mutants. To test this possibility, HEK 293T cells were transfected with equivalent total amounts of DNA containing various groupings of the C66, C84, C101, and C118 MLD mutants. Transduction efficiency into mixed or single MLD mutants, WT, or empty vector populations was plotted against the average MLD length (Fig. 3E).

If synergy was occurring, we would expect that transduction into mixed MLD populations to be equivalent to transduction into cells expressing only the longest MLD mutant from that group. However, we found that the transductions of mixed populations were equivalent to the average transduction of the MLD lengths transfected, suggesting that no synergy was occurring.

The specific sequence of MLD is nonessential. Although a sufficient length MLD is required for TIM-1 enhancement of trans-
duction, we wanted to determine whether specific sequences within the MLD were required in addition to a minimal length. Our first approach to address this question was to replace the 167 aa that compose the TIM-1 MLD with 157 aa of an unrelated MLD from murine alpha-dystroglycan (αDG) to generate the αDG-MLD chimera. This h1PRR chimera also is predicted to contain 35 O-linked glycosylations as detected by NetOGLyc 4.0 Server, about half as many the predicted 67 for WT TIM-1s (30). Expression of this chimera on the surfaces of cells was considerably lower than that of WT TIM-1. In order to examine transduction using cells with comparable surface expression of h1PRR and WT TIM-1, the amount of TIM-1 DNA transfected was reduced until the surface expression of the TIM-1 IgV domain as detected by ARD5 was equal to that of the h1PRR-transfected cells (Fig. 4C).

Structural requirements of the MLD. Protein sequences containing a high frequency of O-linked glycosylations and prolines such as MLDs are believed to form long extended, beta-turn helix conformations (27). Therefore, we hypothesized that the role of the MLD in PVEERs is to provide an extended stalk structure that positions the IgV domain of TIM-1 a sufficient distance from the host cell surface for interaction with virions. In order to test this hypothesis, we replaced the TIM-1 MLD with the well-studied murine leukemia virus (MuLV) SU glycoprotein proline-rich region (PRR) that is not considered to be an MLD but acts as a flexible hinge (28) and likely forms a beta-turn helix (29). Since the PRR is only half the minimal length required for a PVEER MLD, two head-to-tail tandem repeats of the PRR consisting of a total of 142 aa were substituted for the TIM-1 MLD in the h1PRR chimera. This h1PRR chimera also is predicted to contain 35 O-linked glycosylations as detected by NetOGLyc 4.0 Server, about half as many the predicted 67 for WT TIM-1s (30). Expression of this chimera on the surfaces of cells was considerably lower than that of WT TIM-1. In order to examine transduction using cells with comparable surface expression of h1PRR and WT TIM-1, the amount of TIM-1 DNA transfected was reduced until the surface expression of the TIM-1 IgV domain as detected by ARD5 was equal to that of the h1PRR-transfected cells (Fig. 4C).

Next, we sought to determine whether a more structured stalk
of equivalent or longer length would functionally replace the TIM-1 MLD. We previously showed that another PtdSer-binding protein, RAGE, does not enhance virus entry (5). Like TIM-1, RAGE contains an N-terminal IgV domain, but the stalk region of RAGE is composed of two structured Ig-like C2 domains, each composed of more than 90 aa (31). We generated several TIM-1/RAGE chimeras and compared transduction efficiency to that of WT TIM-1. Similar to h1PRR, the TIM-1/RAGE constructs had reduced expression compared to WT TIM-1, and for these mutants we compensated in a similar manner (Fig. 4C). For our initial TIM/RAGE chimera, we replaced the IgV domain of RAGE with that of TIM-1 (h1RAGE). The presence of the TIM-1 IgV domain on the remainder of RAGE minimally enhanced virus entry above that of the empty vector, resulting in ~25% of WT TIM-1 transduction levels. Empty vector transduction data are compared to the same TIM-1 transduction data as h1RAGE and h1RAGEh1cyto since these had the lowest expression levels. (E) Expression of WT TIM-1 and AnxV-αDG-GPI as detected using TIM-1 IgV and MLD specific MAbs ARD5 and AKG7 and AnxV antisera (black line) compared to background IgG binding (filled gray). (F) Transduction of cells expressing either TIM-1 or AnxV-αDG-GPI. Cells were transduced with an MOI of 0.02 (EBOV and LASV in panels B and F) or 0.03 (EBOV and LASV in panel D and all other viruses in panel F) as titered in empty vector transfected HEK 293T cells. The data are shown as means ± the SEM of at least three replicates. Significance was calculated using a two-sample t test comparison to empty vector (B) or a one-sample t test comparison to 100 (D) (**, P < 0.001).

Mimicking functions of TIM-1 domains produces an artificial PVEER. In this and previous studies we individually substituted the IgV domain (5), MLD (Fig. 4B), and cytoplasmic domains of TIM-1 (Fig. 1I) and retained PVEER functionality.
Together, we interpret these results to indicate that PVEER function is derived from the combination of a PtdSer-binding domain linked to the host cell membrane by an MLD of at least 120 residues. If this interpretation is correct, then it should be possible to generate a PVEER chimera without any TIM-1 sequence. We created AnxV-αDG-GPI by attaching annexin V, a PtdSer-binding protein, to a GPI-anchored MLD of αDG. This chimera was detected by an annexin V specific antibody but not TIM-1 specific MAbs (Fig. 4E) and enhanced EBOV pseudovirion entry as effectively as WT TIM-1 (Fig. 4F). In addition, like TIM-1, AnxV-αDG-GPI enhanced entry of VSV pseudovirions bearing Sindbis 2.2 LILV env (SINV) (32–34), Ross River virus (RRV) GP, Chikungunya virus (CHIKV) env, or baculovirus Autographa californica nucleopolyhedrovirus GP64.

**DISCUSSION**

TIM-1 and TIM-4, along with other PVEERs, such as Gas6/Axl, have recently been identified as broad enhancers of virus entry through interaction with PtdSer on the viral envelope (2–4, 8). We and others found that mutations which alter the TIM-1 IgV PtdSer binding pocket decreased virion binding and transduction, providing compelling evidence that the IgV domain is critical for PVEER function (2, 3, 5). However, we confirm here that TIM-3 is poor at enhancing virus entry, as others have found (2–4), despite the conservation of PtdSer-binding activity (12). In the present study, we extended this understanding by characterizing the features of the other TIM-1 domains required for enhancing virus entry and determining those that account for the differences in efficacy among TIM family members. Through extensive analysis of chimeric proteins and deletion mutants, we established several conclusions regarding the characteristics of PVEER domains.

First, neither the cytoplasmic tail nor the transmembrane domain is needed for PVEER function, provided the protein is still membrane bound. Second, a MLD of sufficient length is required for PVEER function and additional length beyond ~120 aa is unnecessary. Third, a specific MLD sequence is not required, and proline/serine/threonine-rich sequences from other proteins can substitute for that of TIM-1 MLD. However, some structural constraints on this region are apparent, as evidenced by the failure of Ig-like C2 domains containing similar numbers of residues to substitute for the TIM-1 MLD. Finally, an artificially generated PVEER that mimics the functional domains of TIM-1 mediates transduction as efficiently as WT TIM-1 even though it lacks sequences from any TIM family member.

The mechanism of TIM-mediated enhancement of virus entry and uptake of apoptotic bodies remains unknown. The cytoplasmic tails of TIM-1 and TIM-3 are functionality important in T cells for signaling through pathways associated with activation of the T-cell receptor signaling complex (18, 23–25, 35). However, these TIM family members have not been reported to be involved with uptake of apoptotic bodies or viruses in T cells, and these signaling events may be required for other T-cell specific functions. Consistent with this possibility, we and others have demonstrated that the intracellular domains of the TIM family members are dispensable for enhancement of virus entry (3). However, we cannot rule out that these domains do contribute during endogenous expression. In addition, our observations that the TIM-1 IgV domain can be replaced with annexin V (5) and the MLD with that of αDG or Pro/Ser/Thr-rich sequences from MuLV Env suggest that specific, direct, intracellular, and extracellular protein-protein interactions are nonessential for TIM family PVEER function. On one hand, our findings are consistent with previous studies that suggest the TIM family members enhance uptake of apoptotic bodies or virus by functioning as attachment factors (2, 3, 20). However, such a model of virion entry must account for the ability of PVEERs to stimulate rapid uptake of viruses lacking a glycoprotein (2, 5). If PVEERs do not directly mediate virus internalization into endosomes, additional receptors or cofactors responsible for internalization must also bind the virion envelope. Alternatively, binding of multiple PVEERs may result in local changes to plasma membrane structure, such as membrane curvature, that induce cargo internalization in response.

Members of the TAM family of receptor kinases (Tyro3, Axl, and Mer) are PVEERs that when complexed with Gas6 enhance binding of virions to cells (3, 8). In contrast to the TIM proteins, the TAM receptors require signaling through their cytoplasmic domains for robust enhancement of virus infection (3, 8, 36, 37). However, this signaling inhibits innate immune responses and has not been shown to directly enhance entry (36). This is supported by the ability of a kinase-dead Axl to enhance virus internalization equivalently to WT Axl (3, 36). Thus, although signaling events of the TAM kinases contribute significantly to infection, signaling likely does not affect virus internalization and therefore are unlikely to contribute to PVEER function, which is consistent with our conclusions for TIM-1.

We would predict that TIM-1 expression in the host has both beneficial and deleterious effects; although TIM-1 is important for immune regulatory functions and clearance of dying and apoptotic cells, it also enhances virus entry. Thus, it is not surprising that high sequence variability and evidence of positive selection within the TIM-1 MLD is found in human populations (38). There is also some evidence for variability within Old World monkeys (39). In both cases, this variation is due to amino acid substitutions or insertions/deletions at multiple sites within the MLD. For instance, in humans, a frequent TIM-1 MLD polymorphism, 157ins/delMTTVP, results in a 6-aa insertion or deletion. However, we and others (2) have found that larger MLD sequence deletions are required for significant effects on TIM-1 uptake of virus than seen within natural variation. Large TIM-1 MLD deletions may have profound consequences for immune function. In mice, deletions within the TIM-1 MLD lead to defects in IL-10 production by B cells and subsequent development of autoimmune disorders (40). This is attributed to the inability of B cells to interact with apoptotic cells and induce IL-10 secretion (41). Even the smaller 157ins/delMTTVP polymorphism found in humans has been associated with autoimmune problems of atopy, eczema, and asthma (38, 42). However, the linkage between these diseases and TIM polymorphisms remains controversial, perhaps due to very modest alteration of MLD length. In total these studies suggest that a MLD of sufficient length is essential for efferocytosis and maintenance of homeostasis. Significant deletions to evade viruses would be expected to come at the price of considerable detrimental effects. Interestingly, however, TIM-1 is nonfunctional in many New World monkeys (39), suggesting either that compensatory changes can overcome these effects or that the function of TIM-1 can differ in some species.

While the specific sequences of the MLD are not essential for enhancement of transduction, some constraints are apparent. Noting that our MLD deletion mutations encompassed about three-quarters of the MLD residues in a region that contains re-
petitive threonine-, serine-, and proline-rich sequences and predicted O-linked glycosylation sites, we chose to construct chimeras using two glycoproteins that lack sequence homology to TIM-1, but are enriched in similar residues, and a third protein, RAGE, that has more condensed structure (Fig. 4A). Although the 225 residues IgC-2 domains of RAGE are longer than the TIM-1 MLD, these sequences fold into rigid and relatively compact beta-barrels stabilized by two disulfide bonds (31, 43). In contrast, while the structures of the TIM-1 and αDG MLDs and the PRR of MuLV have not been solved, these domains are thought to have an extended structure. Interestingly, similar domains were recently shown by using small angle X-ray scatter, circular dichromism, and nuclear magnetic resonance (NMR) analyses to fold into multiple conformations between which they readily interconvert, producing intrinsic disorder that evades structure solution by conventional X-ray crystallography (44). In organic solvents, they fold into extended polyproline II helices in which all of the proline residues are trans, but in water cis isomerization of one or more proline residues creates turns and generates multiple conformers (44), lending a degree of flexibility to shape and size that has been shown to be essential to domain function of several proteins (45–48). In our model (see Fig. 5A), key chimeras are depicted as stalks of extended, rod-shaped, polyproline-like helices. However, we speculate that this is one of many conformations in which these domains can fold and that interconversion provides flexibility to a substantial area pivoting around the carboxy-terminal most proline residues (Fig. 5B).

A longer MLD could provide several benefits for enhancing virus entry over a shorter MLD. The enhanced length may potentially project the PtdSer binding pocket of the IgV, necessary for interaction with PtdSer on the virus, above other cell surface proteins and sugars, thereby reducing steric hindrance (Fig. 5A). This might explain why the additional length of the 2X MLD mutant provides no additional benefit once the IgV has already cleared the majority of cell surface milieu. Similarly, steric hindrance may also be caused by proteins present on the virion surface such as the GP. In addition, the increased length of the MLD might provide a greater area of interaction with the viral membrane by expanding the circumference of rotation around the anchor or transmembrane domain (Fig. 5B). Finally, the longer MLD may provide some flexibility for interaction with and passage of virus to other proteins. Although our studies did not find synergy between MLD of various lengths that would suggest transfer of virus between TIM proteins, flexibility may still allow for multiple TIM molecules to bind to the same viral envelope by bending as needed to the curvature of the viral envelope (Fig. 5C). Consistent with this possibility, both adenoviruses and reoviruses have been shown to require flexibility and length in the stalk of their attachment proteins for receptor binding (49, 50). Taken together, these results suggest that adequate distance and flexibility on either the host or virus side are necessary for effective receptor-virus interactions and infection.

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