Receptor-activated human $\alpha_2$-macroglobulin interacts with the envelope protein of dengue virus and protects virions from temperature-induced inactivation through multivalent binding

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Based on the hypothesis that interactions between virions and serum components may influence the outcome of dengue virus (DENV) infections, we decided to use affinity chromatography with domain III from the envelope (E) protein of DENV2 (DIIIIE2) as a ligand to isolate virus-binding proteins from human plasma. This approach yielded serum amyloid P (SAP) and $\alpha_2$-macroglobulin ($\alpha_2$M) as novel viral interactors. After confirming the specific binding of both SAP and $\alpha_2$M to DIIIIE2 by ELISA, the latter interaction was examined in greater detail. We obtain evidence suggesting that the binding species was actually the receptor-activated form of $\alpha_2$M ($\alpha_2$M*), that $\alpha_2$M* could bind monovalently to recombinant domain III from all four DENV serotypes with affinities in the micromolar range ranking as DENV4 $\approx$ DENV1 $\approx$ DENV2 $\approx$ DENV3 and that this interaction exhibited a strong avidity effect when multivalent binding was favoured ($K_D \approx 8 \times 10^{-8}$ M for DIIIIE2). We also showed that $\alpha_2$M* bound to DENV virions of the four serotypes, protecting the virus from temperature-induced inactivation in the absence of serum and enhancing infectivity. The latter effect exhibited an ED$_{50}$ of $2.9 \times 10^{-8}$ M, also suggesting an avidity effect due to multivalent binding. These results will further contribute to the characterization of the virus–host factor interaction network during human DENV infection.

INTRODUCTION

Dengue, a mosquito-borne disease caused by any of four closely related flaviviruses forming the dengue virus (DENV) complex, has become a global public health problem of growing proportions (Bhatt et al., 2013). With no vaccine yet available against this disease (Thisyakorn & Thisyakorn, 2014), considerable effort has gone into characterizing the cellular DENV interactome in the hope of identifying druggable targets for specific antiviral therapies (Krishnan & Garcia-Blanco, 2014). To the best of our knowledge, however, these efforts have never focused on examining the interactions of DENV virions with serum or plasma proteins.

Plasma and/or serum are the first milieu DENV virions encounter before entry and replication in their target organs. Thus, serum factors may play a pivotal role in determining the outcome of DENV infections. One example is the phenomenon of antibody-dependent enhancement, where pre-existing non-neutralizing DENV-specific antibodies may increase disease severity (Halstead, 2003, 2007). Indeed, viraemia has been correlated with disease severity despite the fact that virus titres drop long before the appearance of life-threatening clinical symptoms (Vaughn et al., 2000) and DENV infections produce qualitative changes in the composition of the plasma proteome, although whether the latter constitutes cause or consequence of disease manifestations remains unknown (Albuquerque et al., 2009; Fragnoud et al., 2012; Houghton-Triviño et al., 2010; Mairuhu et al., 2005; Nascimento et al., 2009).

In DENV virions, the nucleocapsid is surrounded by a lipid bilayer that is almost completely covered by envelope (E) protein dimers, formed by monomers arranged in antiparallel fashion (Kuhn et al., 2002; Mukhopadhyay et al., 2005). Each monomer has a three-domain structure, with a central eight-stranded $\beta$-barrel (domain I) bearing, on one side, two large loops between consecutive strands that together form the finger-like domain II (bearing at its tip the fusion peptide) and, on the other side, a linker connecting to an independently folded immunoglobulin-like domain (domain III) (Modis et al., 2003), which is involved directly in the interaction of DENV with its cell

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surface receptors (Chen et al., 1996; Chu et al., 2005; Crill & Roehrig, 2001) and with potent neutralizing antibodies (Matsui et al., 2010; Sukupolvi-Petty et al., 2007; Thullier et al., 2001).

Domain III can be expressed in *Escherichia coli*, and refolded to a structurally correct and functionally active state (Guzman et al., 2010; Huerta et al., 2008; Hung et al., 2004; Yu et al., 2004). Therefore, in order to identify serum factors that may modulate the course of dengue infections, we used affinity chromatography of whole human plasma with a recombinant domain III preparation from DENV2 as ligand to isolate DENV-binding proteins. The obtained species were identified by MS, confirming their interaction with DIIIIE2 and DENV by ELISA. The possible influence of the identified interactions on the course of viral infection is also discussed.

**RESULTS**

**Isolation of serum proteins interacting with domain III of the E protein of DENV2**

Serum proteins binding domain III of the E protein of DENV2 were isolated by affinity chromatography using the recombinant protein DIIIE2 as immobilized ligand. DIIIE2, comprising residues Met289–Gly400 of the E protein from DENV2, was obtained as a highly pure (Fig. 1a) and correctly folded preparation, as assessed by the binding of neutralizing mAb 3H5 (Fig. 1b), which recognized a topographic epitope on DENV2 domain III (Hiramatsu et al., 1996; Huerta et al., 2008). After coupling DIIIE2 to CNBr-activated Sepharose, the resulting affinity gel (together with a parallel background control) was then used to isolate potential interactors from a conditioned human plasma sample, prepared by pooling non-immune blood donations.

SDS-PAGE analysis of the eluates from these affinity columns revealed the presence of three protein bands migrating above 67 kDa in the DIIIE2 eluate that were absent from the background control eluate (bands a1, a2 and b, Fig. 2). The protein species in bands a1 and a2 was identified as x2-macroglobulin (x2M), and that in band b as the serum amyloid P (SAP) component (Table 1). Protein bands c–f were present in both samples, but with different intensities, and contained human serum albumin (HSA), and IgG and IgM.

x2M is a 720 kDa homotetramer of identical 180 kDa glycoprotein subunits that acts as a proteinase inhibitor against proteases of all mechanistic families. It exhibits a mean plasma concentration of 2 mg ml\(^{-1}\), which sits in the micromolar range of concentration. x2M also has a receptor-activated form (x2M*) which is found only in trace amounts, as it is rapidly eliminated from the bloodstream through receptor-mediated endocytosis via the low-density-lipoprotein receptor-related protein, LRP1 (Imber & Pizzo, 1981; Lillis et al., 2008).

SAP, also a typical plasma component, exhibits mean concentrations in the 30–50 \(\mu\)g ml\(^{-1}\) range (Bottazzi et al., 2010) and is therefore enriched significantly by DIIIE2 affinity chromatography. SAP is a pentameric glycoprotein that consists of five identical subunits of 25 kDa and belongs to the short arm of the pentraxin family of proteins.

**Evaluation of the binding of the isolated proteins to DIIIE2**

The interaction of DIIIE2 with purified preparations of HSA, x2M, x2M* and SAP was investigated by ELISA (Fig. 3), using biotinylated DIIIE2. It was decided to examine binding of x2M and x2M* separately, as the large differences in plasma concentrations exhibited by both species and the rapid interconversion from one form to the other

![Fig. 1. Characterization of the DIIIE2 preparation. (a) Analysis by silver-stained SDS-PAGE (15 %) (Laemmli, 1970). Lanes: 1, molecular mass markers; 2, 12 \(\mu\)g DIIIE2 in reducing sample buffer. (b) Analysis of the binding of anti-DENV antibodies, measured by SPR. RU, resonance (arbitrary) units. Both mAbs were applied at 10 \(\mu\)g ml\(^{-1}\) in HBS-EP. mAb 4G2 was used as negative control.](http://vir.sgmjournals.org)
through cleavage of the bait region might have important pathophysiological implications. The purity of the α2M and α2M* preparations used in this experiment was assessed by non-denaturing PAGE, which yielded the expected banding pattern (faster migration for methylamine-treated α2M, indicating that the conformational change associated with the formation of the receptor-activated form of the protein had taken place).

Biotinylated DIIIE2 bound to SAP, α2M and α2M* in a dose-dependent manner, indicating a specific interaction. The ED50s of biotinylated DIIIE2 binding to α2M and α2M* were very close (1.6 ± 10^{-6} and 1.3 ± 10^{-6} M, respectively) (Fig. 3), indicating interactions of very similar affinities. However, for α2M, in contrast to α2M*, binding reached saturation at very low signal strengths. Considering the ease with which α2M converted to α2M* in the presence of proteases, this result suggested that the α2M binding curve actually signalled the existence of small amounts of α2M* in this preparation, perhaps due to trace protease contaminants. There was some overlapping of the protein bands corresponding to α2M and α2M* in PAGE (inset, Fig. 3), which was indeed consistent with this possibility.

**Table 1. Summary of peptide sequences and protein identification**

<table>
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<th>Gel band</th>
<th>Peptide sequence</th>
<th>UniProt accession no.</th>
<th>Description</th>
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<td>911LPPNVVEESAR21</td>
<td>P01023</td>
<td>Human α2M</td>
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<tr>
<td>b</td>
<td>563VTAAPQSVCALR24</td>
<td>P02743</td>
<td>Human SAP component</td>
</tr>
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<td>85VGEYSLYIGR96</td>
<td>P04220</td>
<td>Heavy chain from human IgM</td>
</tr>
<tr>
<td>d</td>
<td>151VLQGEQDSYGK162</td>
<td>P02768</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>e</td>
<td>78LIQATGESP99</td>
<td>P01859</td>
<td>Heavy chain from human IgG</td>
</tr>
<tr>
<td>f</td>
<td>62DSTYLSLTLSDK75</td>
<td>P01834</td>
<td>Light chain from human IgG, constant region</td>
</tr>
</tbody>
</table>

*Corresponds to the identification of gel bands in Fig. 2.

![Fig. 2. Analysis by 12.5% SDS-PAGE (Laemmli, 1970) of DIIIE2-binding proteins obtained by affinity chromatography. Lanes: 1, molecular mass markers; 2, human plasma used as initial sample; 3, 4, flow-through fractions; 5, 6, eluates from the background control and DIIIE2 columns. The protein bands were visualized with a modified silver-staining protocol compatible with downstream MS analysis (Jensen et al., 1999). The species selected for further identification by MS are indicated with a letter. Different bands sharing the same letter correspond to the same species identified in MS.](image)

![Fig. 3. Evaluation by ELISA of the interaction of DIIIE2 with the proteins identified in the affinity chromatography eluate. Data correspond to the mean with range from two independent experiments, each performed in triplicate. Lines correspond to the log10[agonist] versus response fits performed with GraphPad Prism v.5.3 used for the calculation of the ED50 values (shown in the table). Inset: silver-stained non-denaturing PAGE (5% gel) analysis of (1) α2M and (2) α2M*.](image)
ED₅₀ for the binding of biotinylated DIIIE₂ to SAP in this assay also fell in the micromolar range (6.0 ± 10⁻⁶ M), indicating, as in the case of α₂M*/α₂M, a relatively weak interaction. No binding of biotinylated DIIIE₂ to HSA was detected.

**Interaction of α₂M* with DENV virions**

Two different experimental approaches were followed in order to determine whether α₂M* interacted with DENV virions. First, we assessed the binding of α₂M* to virions from all four DENV serotypes by capture ELISA. In this case, α₂M* exhibited specific binding to DENV virions independently of their serotype (Fig. 4).

The second approach consisted of examining the impact on virus infectivity of pre-incubating α₂M* with viral preparations at 37 °C. As this experimental setup required serum-free conditions, and in light of anecdotal reports suggesting that incubation at 37 °C in such conditions may decrease virus infectivity, we conducted preliminary time-course experiments to control for this factor. Under our experimental parameters, incubating DENV virions at 37 °C in the absence of FBS did lead to a progressive, time-dependent decrease of virus infectivity (Fig. S1a, available in the online Supplementary Material). This effect was more pronounced at lower m.o.i. (i.e. m.o.i. 0.001; Fig. S1b), probably due to the progressively lower concentration of residual FBS in the diluted viral stocks.

Fig. 5 depicts the result of the pre-incubation assay run either with α₂M*, at dilutions starting from 1.5 mg ml⁻¹ (2.1 × 10⁻⁶ M), or with HSA at equimolar concentrations. Under these conditions, at m.o.i. 0.001, the presence of α₂M* prevented DENV2 inactivation in a dose-dependent manner, with an ED₅₀ of 2.9 × 10⁻⁸ M. Also at m.o.i. 0.001, and when α₂M* concentrations were >10⁻⁷M, a trend to increase virus yield to an amount that represented up to 150% compared with virus control kept at 4 °C was apparent. At higher m.o.i. the effect of thermal inactivation of the virus was less significant (m.o.i. 0.01, Fig. 5b) or non-existing (m.o.i. 0.1 and 1; Fig. S2); thus the protection effect was not relevant under these infection conditions. The increase in virus yield was also not detectable at higher m.o.i. HSA exhibited a similar behaviour, although the increase in virus yield caused by this protein seemed to reach a maximum considerably lower than that obtained with α₂M*.

However, at physiological concentrations HSA actually decreased virus infectivity (Fig. S3). No protective effect was obtained by pre-incubating α₂M* or HSA with the cells for 45 min at 37 °C before infection or by adding the test protein and the viral inoculum simultaneously just before infection (data not shown). These results suggested that HSA interacted directly either with regions of the E protein other than DIII or with a different DENV structural protein.

**Characterization of the interaction of α₂M* with DIIIE₂ by surface plasmon resonance (SPR)**

The interaction of α₂M* with DIIIE₂ was also characterized by SPR (Fig. 6a). DIIIE₂ was immobilized by amine
coupling chemistry, yielding a surface with 250–350 RU (resonance units). Preliminary experiments at different flow rates (5–50 μl min⁻¹) yielded superimposed sensorgrams, evidencing the absence of mass transfer limitations at this ligand density and indicating that this surface was adequate for the study of kinetic constants. The data from all curves fitted a Langmuir 1:1 model very well, with good randomization of residuals (Fig. 6a), and a $K_D$ value for the interaction of DIIIE2 and $x_2M^*$ of $8 \times 10^{-8}$ M. Although this value contrasts with the ED₅₀ constants determined by ELISA, it must be remembered that in this SPR experiment the analyte ($x_2M^*$) was a homotetramer that could bind several immobilized DIIIE2 molecules at once. Multivalent binding was, in fact, the simpler explanation for this discrepancy, which turns the obtained value into an apparent affinity constant.

Data on the relative affinity of the interaction of $x_2M^*$ with domain III of the E protein from different DENV serotypes was also obtained by means of a competition ELISA (Fig. 6b). Recombinant domain III from strains of serotypes 1, 3 and 4 of DENV were obtained by heterologous expression in E. coli and purified to homogeneity (inset, Fig. 6b). Using a fixed concentration of biotinylated DIIIE1 protein, binding to $x_2M^*$-coated wells was measured in the presence of increasing concentrations of competing DIIIE2, DIIIE3 and DIIIE4. The specificity of the interaction was verified by using $x_2M^*$ and DIIIE1 as competing ligands, which exhibited IC₅₀ values of $1.1 \times 10^{-6}$ and $4.1 \times 10^{-6}$ M, respectively (Fig. 6b). No competition was detected across the entire range of evaluated protein concentrations when using HSA as competing ligand. DIIIE2 and DIIIE4 blocked DIIIE1 binding, although DIIIE4 exhibited a 10-fold lower IC₅₀ ($2.2 \times 10^{-7}$ M) than DIIIE2 ($2.4 \times 10^{-6}$ M). Notably, although DIIIE3 exhibited an IC₅₀ of $1.5 \times 10^{-6}$ M, it only blocked binding of DIIIE1 to $x_2M^*$ by at most 40%, even at the highest concentrations.

**DISCUSSION**

Affinity chromatography has been used previously to fish for proteins interacting with either DENV viral particles (Moreno-Altamirano et al., 2002) or a recombinant E protein ectodomain (Reyes-del Valle & del Angel, 2004; Reyes-del Valle et al., 2005). Both studies, which used cell extracts as the source of interacting partners, led to the identification of putative virus receptors. Here, we used affinity chromatography with a recombinant domain III from DENV2 (DIIIE2) as ligand to identify instead potential DENV interactors in human plasma. This approach resulted in the isolation and identification of two different proteins as potential novel interaction partners of DENV2 in human plasma: $x_2M/x_2M^*$ and SAP (Fig. 2, Table 1).

Our results point to $x_2M^*$ (i.e. receptor-activated $x_2M$), but not $x_2M$, as the actual interactor with domain III of the E protein of DENV2 (Fig. 3), suggesting that this interaction involves surfaces created or unmasked upon $x_2M$ activation. A direct interaction of $x_2M^*$ with all four serotypes of DENV was also demonstrated (Figs 4 and 6). Although characterized in lesser detail in the present work, an interaction of SAP with domain III of the E protein of DENV2 was also evidenced.

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**Fig. 6.** Interaction of $x_2M^*$ with recombinant domain III preparations from all four DENV serotypes. (a) SPR analysis. Purified $x_2M^*$ was applied in different dilutions: (A) 1.3, (B) 1, (C) 0.63, (D) 0.3 and (D) 0.16 μM. (b) Competition ELISA for binding to $x_2M^*$-coated plates. Biotinylated DIIIE1 was used at 0.75 μg ml⁻¹ ($6.6 \times 10^{-8}$ M) in the presence or absence (maximum binding) of competitor. The legend shows the ED₅₀. Inset: SDS-PAGE analysis (15% gel) of the purified domain III preparations: (1) DIIIE1, (2) DIIIE2, (3) DIIIE3 and (4) DIIIE4. Data represent the mean with ranges from two independent experiments, each performed in triplicate. The gel was stained with Coomassie blue.
Two different studies have shown alterations in the plasma levels of $x_2M/x_2M^*$ in DENV-infected individuals (Albuquerque et al., 2009; Kumar et al., 2012). However, their results are mutually contradictory. Our data provide a plausible explanation for the apparently contradicting results of these two publications, as neither one differentiates between the two forms of the protein, i.e. $x_2M$ and $x_2M^*$.

Binding of $x_2M/x_2M^*$ to its different protein ligands has been shown to involve different mechanisms, including entrapment into its cage-like structure but also covalent binding through its reactive thiol ester bond and non-covalent binding (Borth, 1992). Our results indicate that the interaction of $x_2M^*$ with DENV is reversible and takes place through binding to domain III of the viral E protein, although the possible existence of additional interaction sites in the E or membrane (prM/M) proteins of DENV virions cannot be ruled out.

Both the ED$_{50}$ for biotinylated DIIIIE2 binding monovalently to surface-immobilized $x_2M^*$ and the IC$_{50}$ for unmodified domain III from different serotypes competing with biotinylated DIIIE1 for binding to surface-immobilized $x_2M^*$ (Figs 3 and 6b) fall within the micromolar range, indicating that this is a weak interaction, unlikely to take place at the low physiological concentrations exhibited by $x_2M^*$. (The agreement between these magnitudes, in addition, indicates that biotinylation does not impair significantly the binding of domain III to $x_2M^*$. However, the interaction is 20-fold stronger when measured under conditions where $x_2M^*$ may bind multivalently to surface-immobilized DIIIIE2 ($8 \times 10^{-8}$ M apparent affinity constant, 20-fold stronger, Fig. 6a). Although differences in assay format (ELISA versus SPR) and domain III modification (biotinylation versus amine coupling) preclude invoking multivalent binding as the sole cause for this discrepancy, the fact that the ED$_{50}$ for the stabilization of DENV virions by unmodified $x_2M^*$ in solution ($2.9 \times 10^{-8}$ M, at least 50-fold stronger, Fig. 5a) has the same order of magnitude as the SPR $K_D$ supports the interpretation of the existence of avidity due to multivalency. In fact, the particular arrangement of domain III in the three- and fivefold symmetry axes of the virion probably favours the multivalent interactions of higher avidity observed in this case (Kuhn et al., 2002; Watterson et al., 2012), making them more likely to be physiologically relevant.

Two groups have recently uncovered important changes in the structure of DENV particles produced in mosquito (C6/36) cells upon incubation at temperatures >35 °C (Fibriansyah et al., 2013; Zhang et al., 2013). In both cases, it was demonstrated that the virus changes from a compact form exhibiting icosahedral symmetry to expanded and less-organized variants that remain equally infective in BHK21 cells. In our hands, however, incubating DENV2 at 37 °C in the absence of FBS caused a significant loss of infectivity. Interestingly, at m.o.i. 0.001, the presence of $x_2M^*$ not only protected DENV2 from this temperature-induced inactivation, but increased infectivity up to 150% compared with the non-incubated control. A lower susceptibility to infection enhancement at m.o.i. 1, compared with the at least threefold obtained at m.o.i. 0.001 and 0.1, has been observed for the antibody-mediated potentiation of the infection obtained in cells bearing Fcγ receptors (Halstead, 2003). In our results, however, the infection enhancement is less pronounced and is observed only at m.o.i. 0.001, which points to this condition as being close to the infection threshold for DENV2 in Huh7.5 cells. Thus, taking into account the multivalent $x_2M^*$–DENV interaction suggested by our data, we believe that both the protective effect upon thermal inactivation as well as the enhanced infection can be interpreted as the consequence of restrictions imposed on the structural changes of virus particles when complexed with $x_2M^*$. Our results indicate that such structural stabilization of the virus not only does not impair the interaction with any cell surface receptor(s), but can be favourable under conditions of a challenging low dose of infection.

Even though domain III is thought traditionally to exhibit serotype-specific reactivity exclusively, several complex-reactive epitopes targeting this region have also been identified (Hung et al., 2004; Thullier et al., 2001; Watterson et al., 2012). The results of the binding competition experiment indicate that the $x_2M^*$ interaction site is shared across domain III molecules from all four DENV serotypes, despite the presence of important differences in binding affinity for serotype 4 (Fig. 4b). Likewise, the data also suggest that this interaction involves more than one contact site, as indicated by the partial inhibition obtained with the DIIIE3 competitor.

Further research is needed to fully elucidate the significance of the interactions with $x_2M^*$ and SAP for DENV infection in vivo. On the one hand, $x_2M^*$ binds protein Grp78, which has been shown to act as an $x_2M^*$ coreceptor together with LRP1 (Misra et al., 2002). On the other hand, Grp78 has been identified as a putative receptor for DENV2 in the hepatoma-derived cell line HepG2 (Jindadamrongwech et al., 2004), even though the available evidence indicates that virus entry also involves an endocytic receptor. Putting the above together, it follows that future experimentation must address the potential role of $x_2M^*$ as a shuttle for DENV to Grp78–LRP1 as a receptor–coreceptor complex that may mediate virus endocytosis.

Our finding that SAP can be isolated by affinity purification of whole plasma with DIIIIE2 bears similar significance. As with other pentraxins, SAP binds a great variety of ligands, and has been shown to activate complement and mediate the phagocytosis of apoptotic cells and microorganisms via direct binding to Fcγ receptors (Bottazzi et al., 2010). Taking into account that the binding of complexes of DENV with non-neutralizing IgG antibodies to Fcγ receptors constitutes the molecular basis of antibody-dependent enhancement of DENV infection and disease (Halstead, 2003), it follows that examining whether binding of non-IgG molecules (like SAP) to Fcγ receptors may also
modulate DENV infection constitutes another potentially rewarding research avenue.

Finally, the phenomena described here, where two different proteins (\(\alpha_2M^*\) and HSA) at physiological concentrations have opposite effects for viral infection in vitro, further reinforce the idea that the outcome of dengue infections probably depends on the balance of an intricate network of interactions with host factors and highlight the need for a more extensive characterization of the DENV plasma interactome.

**METHODS**

**Cells, proteins and viruses.** Vero cells (011038) and DENV 2 (S16803) were obtained from the National Institute for Biological Standards and Control. Vero and Huh7.5 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 U penicillin ml\(^{-1}\) and 100 \(\mu\)g streptomycin ml\(^{-1}\), adding heat-inactivated FBS to a final concentration of 5% (v/v) for Vero and 10% (v/v) for Huh7.5.

Recombinant HSA and BSA were purchased from Sigma-Aldrich. SAP was purchased from Calbiochem. Purified flavivirus-reactive mAb 4G2 and DENV2-specific mAb 3H5 were obtained from the Unit for the Production of Monoclonal Antibodies at the Center for Genetic Engineering and Biotechnology, Sancti Spiritus, Cuba.

Frozen human plasma samples were obtained from expired stocks at local blood banks and screened using a virus capture ELISA to select those without DENV-specific antibodies (data not shown).

**Recombinant domain III from the E proteins of DENV1–4.** Plasmids coding for domain III of DENV1–4 fused to a C-terminal His\(_6\)-tag were prepared by chemical synthesis or PCR amplification of DIII-coding inserts, which after NdeI/XhoI (DIIIE1/DIIIE2) or Ncol/I (DIIIE3/DIIIE4) digests were cloned into the corresponding sites of either pET22b+ (DIIIE1/DIIIE2) or pET28a+ (DIIIE3/DIIIE4) (Table 2). All constructs were verified by DNA sequencing.

After transformation into *E. coli* BL21(DE3) (Studier & Moffatt, 1986) and culture/induction in ZYM5052 medium (Studier, 2005), all four recombinant domains III were obtained as insoluble inclusion bodies that were solubilized into 6 M GuHCl in PBS, refolded by dilution into PBS at 25 \(\mu\)g ml\(^{-1}\) and purified by affinity chromatography on Ni-NTA agarose (Qiagen). In the case of DIIIE4, the refolding buffer consisted of PBS, 0.05% (v/v)/Tween 20 (PBS-T). All buffers contained 1 \(\mu\)g leupeptin ml\(^{-1}\), 1 \(\mu\)g pepstatin A ml\(^{-1}\) and 1 mM PMSF.

The purified proteins were biotinylated as described previously (Huerta et al., 2008).

**Purification of human \(\alpha_2M\).** Human \(\alpha_2M\) was purified from pooled citrate plasma as described previously (Imber & Pizzo, 1981). Briefly, the sample was clarified by centrifugation at 10,000 \(g\) for 30 min, and then the supernatant was dialysed against PBS, pH 6 and subjected to Zn\(^{2+}\) chelate affinity chromatography. All buffers contained the aforementioned protease inhibitor cocktail. The protein was eluted with 10 mM sodium acetate buffer, pH 5, 150 mM NaCl and further purified by gel filtration using a Superdex 200 column (Amersham). Receptor-activated \(\alpha_2M^*\) was prepared by dialysing against 200 mM methylamine in 50 mM sodium phosphate, 150 mM NaCl, pH 7.4 for 18 h at 25°C, confirming activation by analysis in non-denaturing PAGE (5%) as described previously (Imber & Pizzo, 1981).

**Protein immobilization into a chromatographic gel.** Purified DIIIE2 was dialysed against 0.1 mM NaHCO\(_3\), pH 8.3, 0.5 mM NaCl and incubated with CNBr-activated Sepharose (Amersham) for 2 h at 25°C. The uncoupled protein was removed from the gel by centrifugation at 500 \(g\) for 5 min and all remaining reactive groups were blocked with 1 mM ethanamine, pH 8. An identical volume of background control gel was prepared by blocking reactive groups of the CNBr-activated Sepharose with 1 mM ethanamine, pH 8.

**Affinity chromatography.** Human plasma was mixed in a 4:1 ratio with 100 mM HEPES, pH 7.0, 1.75 M NaCl, 25 mM CaCl\(_2\), 5 mM MgCl\(_2\) and loaded into a column packed with the DIIIE2 affinity resin, at a flow of 10 cm h\(^{-1}\). The sample was recirculated on the column for an additional 4 h and then the column was washed with 100 column volumes of 20 mM HEPES, pH 7.0, 0.35 M NaCl, 5 mM CaCl\(_2\), 1 mM MgCl\(_2\). Bound protein was eluted with 10 mM Gly, pH 2.5. A 100 \(\mu\)l aliquot of the collected fraction was precipitated with 10% TCA, resuspended in 20 \(\mu\)l of sample buffer and subjected to SDS-PAGE. Selected protein bands were excised and digested with trypsin followed by electrospray ionization (ESI)-MS analysis of the eluted peptides as described previously (González et al., 2003).

**Analysis by MS.** ESI-MS spectra were acquired using a QTof-2 (Micromass) fitted with a Z-spray nanoflow electrospray ion source operated at 80°C with a drying gas flow at 50 l h\(^{-1}\). Peptides eluted with 60% acetonitrile in 1% formic acid from ZipTips (Millipore) were loaded into the borosilicate nanoflow tips, and submitted to capillary and cone voltages of 900 and 35 V, respectively. Acquisition of ESI-MS/MS spectra proceeded by using the first quadrupole to select the precursor ion within a window of ±3 m/z units. Argon gas was used in the collision chamber at ~3 × 10\(^{-6}\) Pa pressure and collision energies in the range 20–30 eV were set to fragment precursor ions. Data acquisition and processing were performed with the MassLynx v.3.5 (Micromass) package.

**Table 2. Recombinant DIIIE domain data**

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**Peptide identification.** MS/MS data were searched using the Homo sapiens subset from the National Center for Biotechnology Information non-redundant (NCBI) database, using the Mascot search engine (v.2.3.02) with tolerance parameters set to 2 and 0.8 Da for product ion and precursor mass, respectively. Other parameters used were: tryptic peptides with up to one missed cleavage allowed and carbamidomethylation of Cys residues as fixed modification.

**SPR analysis.** Experiments were performed using a Biacore X unit. DIIIIE2 was immobilized covalently to a CM5 chip by amine coupling with 1:1 mixture of 0.1 M pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005 % Polysorbate 20 (HBS-EP) as running buffer. Briefly, the surface of the chip was activated for 7 min with a 1:1 mixture of 0.1 M N-hydroxysuccinimide and 0.1 M N-ethyl-N’-(3-dimethylamino-propyl) carbodiimide, after which protein DIIIIE2 was dissolved at 10 μg ml⁻¹ in 10 mM sodium acetate, pH 4.5, and immobilized at a density of 400 RU. All remaining free activated groups were blocked by the application for 7 min of 1 M ethanolamine, pH 8. Flow channel 2 received only the activating and blocking applications, in order to be used as a reference surface. The experiments were performed at a flow rate of 25 μl min⁻¹, using HBS-EP as running buffer. The interacting surface was regenerated with a 5 μl pulse of 10 mM NaOH.

The determination of equilibrium affinity constants proceeded by loading in duplicate five different concentrations of α2M⁺, injected in random order. Data analysis was performed with BIACevaluation vv.4.1 software (Biacore).

**Virus infection.** Ninety-six-well plates were seeded with 3 x 10⁴ Huh7.5 cells per well and incubated for 24 h at 37 °C, 5 % CO₂. The viral inoculum was diluted in DMEM without FBS and added to cells at m.o.i. 0.001. Virus/protein mixtures were incubated for 45 min at 37 °C before adding them to the cells (a virus aliquot kept for the same period at 4 °C was used as 100 % infectivity control in this case). Infection was allowed to proceed for 2 h, after which the cells were washed three times. Next, 100 μl DMEM with 2 % FBS was added to the cells, which were incubated for 24 h at 37 °C, 5 % CO₂. Virus titres in 24 h post-infection supernatants were determined by plaque formation assays (Morens et al., 1983). The experiment was performed three times independently and each experimental point was assayed in duplicate.

**ELISA.** Ninety-six-well microtitre plates were coated for 1 h at 37 °C with 50 μl per well of the corresponding protein at 10 μg ml⁻¹ in carbonate buffer, pH 9.6. The plates were blocked with 5 % (w/v) BSA in PBS-T for 1 h at 37 °C and washed three times after each incubation step. Biotinylated proteins (or mixtures with competitors) were added and incubated for 1 h at 37 °C, detecting binding afterwards with a streptavidin–HRP conjugate.

Binding of α2M⁺ to viral particles was evaluated with plates coated with 1 μg per well of mAb 4G2. At day 5 post-infection, supernatants from Vero cells infected with DENV1–4 (or non-infected negative controls) were then added and viral capture was allowed to proceed for 18 h at 4 °C. Purified α2M⁺ was diluted at 0.05 mg ml⁻¹ (7 x 10⁻⁸ M) in 20 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM EDTA, 0.05 % Tween 20 (HBS-CaT), added to the plates and incubated for 1 h at 37 °C. Virion-bound α2M⁺ was detected with an anti-α2M⁺ rabbit polyclonal antiserum, followed by an anti-rabbit mAb-phosphatase conjugate.

**REFERENCES**


