RESIDUES IN DOMAIN III OF THE DENGUE VIRUS ENVELOPE GYCOPROTEIN INVOLVED IN CELL-SURFACE GLYCOSAMINOGLYCAN BINDING

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The dengue virus (DENV) envelope (E) protein mediates virus entry into cells via interaction with a range of cell-surface receptor molecules. Cell-surface glycosaminoglycans (GAGs) have been shown to play an early role in this interaction, and charged oligosaccharides such as heparin bind to the E protein. We have examined this interaction using site-directed mutagenesis of a recombinant form of the putative receptor-binding domain III of the DENV-2E protein expressed as an MBP (maltose-binding protein)-fusion protein. Using an ELISA-based GAG-binding assay, cell-based binding analysis and antiviral-activity assays, we have identified two critical residues, K291 and K295, that are involved in GAG interactions. These studies have also demonstrated differential binding between mosquito and human cells.

INTRODUCTION

Dengue virus (DENV) belongs to the medically important genus Flavivirus. Spread by the mosquito vector Aedes aegypti, DENV has re-emerged as the most globally significant arthropod-borne viral pathogen in terms of annual incidence and morbidity. Antiviral and vaccine development against DENV is therefore a priority (Mackenzie et al., 2004). There are four distinct serotypes of DENV (DENV-1–DENV-4) and reinfection with a heterologous serotype is associated with an increased risk of severe disease, thereby complicating effective vaccine strategies (WHO, 2009).

Flaviviruses are small (50 nm), positive-strand RNA, enveloped viruses with a smooth, spherical morphology. The mature virion comprises three structural proteins: a capsid protein (C), which associates with the viral RNA genome; a membrane protein (M), which is derived from a structural precursor (prM); and the glycosylated envelope protein (E), which is the major surface protein and mediates both cell attachment and fusion of the viral and cellular membranes. The atomic structure of E has been solved for tick-borne encephalitis virus (TBEV), DENV-2, DENV-3, West Nile virus (WNV) and Japanese encephalitis virus (JEV) by X-ray crystallography of recombinant proteins comprising the first approximately 400 residues of E, referred to as the soluble E ectodomain (sE) (Kanai et al., 2006; Modis et al., 2003, 2005; Rey et al., 1995; JEV E, PDB accession no. 3P54). This recombinant lacks the C-terminal membrane-spanning anchors and the associated ‘stem’ region. These structural studies demonstrated a conserved global fold across the entire family, suggesting a shared mode of action with respect to the two functional roles of receptor binding and membrane fusion. The structures also revealed that the E glycoprotein forms a head-to-tail homodimer and is composed of three distinct domains, referred to as domains I (DI), II (DII) and III (DIII), that reflect the earlier determined antigenic domains (Guirakhoo et al., 1989). DIII consists of the C-terminal region (residues 292–395) of sE, which adopts an immunoglobulin-like fold of seven anti-parallel β-sheets linked by flexible loops (Huang et al., 2008; Mukherjee et al., 2006; Volk et al., 2004, 2006, 2007; Wu et al., 2003, 2005; Yu et al., 2004). DIII is linked to the central DI via a flexible stretch of amino acids (residues 291–301) that mediate a large inter-domain rearrangement between the pre- and post-fusion forms of E that is thought to drive the fusion process (Modis et al., 2004). DI, an eight-stranded β-barrel, is made up of three segments (residues 1–51, 134–195 and 284–291) interspersed with regions encoding DII. DII contains the majority of residues involved in dimeric interactions and also houses the hydrophobic fusion peptide that initiates membrane fusion via insertion into the cellular membrane. Fitting of the atomic structures onto cryo-electron microscopic reconstructions of whole viruses demonstrated that the mature virion contains 90 E homodimers (Kuhn et al., 2002). The dimers are arranged into rafts in a quasi-T=3 symmetry on the surface of the icosahedral virion, accounting for the unusually smooth appearance of the mature flavivirus virion. This arrangement results in the loops of DIII forming the most distal
Projections from the virion surface, ideally positioned for interaction with a host-cell receptor. Antibody neutralization- and peptide-based studies have further supported a critical role for DIII in host-cell binding (Abd-Jamil et al., 2008; Hiramatsu et al., 1996; Lin et al., 1994; Roehrig et al., 1998; Thullier et al., 2001). In addition to these studies, investigation of sequence variation among cell culture-adapted, serially passed and mutant viruses has also supported a receptor-binding role for DIII (Erb et al., 2010; Lee & Lobigs, 2000, 2002; Lee et al., 2004).

Although no definitive host-cell receptor has yet been identified, a large number of binding molecules on the surface of a range of cell types have been reported in the literature including DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin) (Navarro-Sanchez et al., 2003; Tassaneentithap et al., 2003), laminin receptor (LAMR1) (Thepparit & Smith, 2004; Tio et al., 2005), mannose receptor (Miller et al., 2008), GRP78 (Jindadamrongwech et al., 2004; Upanan et al., 2008), heat shock protein (HSP)70 and HSP90 (Reyes-del Valle et al., 2005) and the αVβ3 integrin (Zhang et al., 2007). Due to the lack of experimental tools, the majority of these studies have focused on the receptor molecules required for DENV entry into mammalian cells. Direct comparison of the routes of virus entry and host machinery involved between mosquito and mammalian cell lines and among the four serotypes has yielded inconsistent results, with some investigations demonstrating divergent entry mechanisms while others report conserved entry pathways. The possibility that DENV uses alternative receptors among the four serotypes and for different cell types, between both the mosquito and the human host and even for tissue-specific cell types therefore remains a plausible hypothesis.

Despite uncertainty regarding the identity of the primary DENV receptor(s), it is now well established that charged cell-surface polymers of sulphated disaccharides known as glycosaminoglycans (GAGs) play a key role in the initial binding of DENV to the cell surface and subsequent entry into mammalian cells. It has been proposed that this interaction serves to localize and concentrate the infectious virions within close proximity of the primary receptor (Putnak et al., 1997); alternatively, the interaction may involve specific GAG groups on the primary receptor itself, as has been observed in other receptor–ligand interactions (Ruiz-Säenz et al., 2009; Vlasak et al., 2005). GAGs are a diverse group of macromolecules and are involved in a wide range of host-cell roles, including cell motility, adhesion, proliferation and development. Due to their high prevalence at the cell surface on nearly all tissue types, it is not surprising that a large number of pathogens including viruses, such as human immunodeficiency virus (de Witte et al., 2007), herpes simplex virus (Akhtar & Shukla, 2009) and respiratory syncytial virus (Hallak et al., 2001), as well as many bacterial pathogens, have evolved to interact with GAGs in order to gain entry into the host cell. GAG binding has also been demonstrated in a cellular context for numerous flaviviruses, including DENV, JEV, yellow fever virus (YFV) and TBEV (Chen et al., 1997; Germi et al., 2002; Kroschewski et al., 2003; Su et al., 2001).

At an atomic level, electrostatic interactions between the negatively charged sulphate groups within GAGs and solvent-exposed basic residues such as lysine and arginine on the protein ligand are thought to mediate the molecular recognition process (Hileman et al., 1998). A number of studies have demonstrated adaptive mutations to acidic amino acids in serial passaging of flaviviruses under cell-culture conditions, with these changes thought to play a role in facilitating enhanced GAG binding (Lee & Lobigs, 2002; Lee et al., 2006). Rather than focus on virus adaptation in tissue culture, we have used a site-directed mutagenesis approach to elucidate which of the naturally occurring, conserved lysine residues within a putative GAG-binding motif in DIII are critical to this interaction. Our results provide a basis for further understanding the interactions of flaviviruses and GAGs on host-cell surfaces.

RESULTS

Design, production and purification of DIII constructs

Two putative GAG-binding regions within sE have previously been identified (Chen et al., 1997), the first (region 1) corresponding to the flexible linker between DI and DIII in conjunction with residues within the β-sheet A strand of DIII. The second binding motif (region 2) lies at the C-terminal end of sE and is therefore not readily accessible in the mature virion (Fig. 1c; K393 and K394). We reasoned that the solvent-exposed nature of region 1 made it a better candidate for the main focus of our mutagenesis strategy; however, we also designed two region 2 deletion mutants of residue K294 and a double deletion of K293/K294 to test this hypothesis. Five highly conserved lysine residues within region 1 (Fig. 1a) were selected for alanine mutagenesis. Of these residues, K291 and K295 are almost completely conserved among pathogenic flaviviruses (Fig. 1b). Residues that were identified as important via the alanine screen using the GAG-binding ELISA were subsequently mutated to arginine to determine the role of electrostatic charge. High level (20–50 mg l⁻¹) expression of all maltose-binding protein (MBP)–DIII fusion-protein constructs was observed with all recombinant proteins present within the soluble fraction after cell lysis. Following purification, soluble DIII was obtained via factor Xa (FXa) digestion of the fusion protein and ion-exchange chromatography (Fig. 1d).

GAG-binding ELISA

Prior to performing cell-based binding studies, we established a high-throughput quantitative in vitro assay for GAG binding. A GAG-based ELISA was developed using BSA-conjugated heparin as a substrate (Nishioka et al., 2007). Binding of our wild-type (WT) construct was
demonstrated with non-conjugated BSA used as a negative control (Fig. 2). The ELISA protocol was then optimized for various conditions, including divalent metal ion and pH dependence. The interaction was shown to be pH-sensitive, with optimal binding at pH 6.5 (Fig. 2a). All subsequent assays were performed at the more physiologically relevant pH 7 without significant loss of binding. No binding of MBP alone was observed at any concentration tested (Fig. 2b), indicating that DIII contained the GAG-interaction motif(s).

To confirm that the observed binding was specific, we performed competitive ELISAs using a constant concentration of MBP–DIII while varying the concentration of the competitor. Soluble DIII was observed to have an inhibitory effect on binding of MBP–DIII at approximately equimolar concentrations, while MBP was not observed to affect binding of MBP–DIII at any concentration tested (Fig. 2c). The specificity of the GAG interaction was investigated using soluble GAGs in a competitive format. Of the soluble GAGs tested, only heparin was shown to have a competitive effect on MBP–DIII binding (Fig. 2d).

**GAG binding of recombinant DIII proteins**

Binding constants of mutant proteins were quantified via GAG ELISA, using serial dilution of the recombinant proteins and determining the dissociation constant ($K_d$) from a saturable one-site model. Among the single alanine mutations, reduced binding relative to WT was only observed for residues K291 and K295 (Fig. 3a). Deletion of region 2 residues K393 or a double deletion of both K393 and K394 did not significantly alter the binding of these recombinant proteins compared with the WT. Combination mutagenesis of lysines in region 1 was then carried out. Only combination mutants containing changes to either K291 or K295 were shown to have reduced binding (Fig. 3b–d). Mutants containing both K291 and K295 mutations consistently demonstrated reduced binding compared with either mutation alone. Conservative mutations of residues K291 and K295 to arginine demonstrated WT-like binding affinity (Fig. 3d).

**Cell binding of recombinant DIII proteins**

Binding of recombinant proteins to human liver (Huh7) and mosquito (C6/36) cells was then analysed with an on-cell far-Western protocol (Fig. 4). Binding of WT and mutant recombinant proteins was determined via recognition of the MBP-fusion partner with anti-MBP rabbit sera, and subsequent indirect binding by a fluorophore-labelled secondary probe was then quantified. For both cell lines, no binding of MBP alone was observed, indicating that binding of MBP–DIII was mediated by DIII. All single alanine mutants were assessed for binding to both cell types. Interestingly, the binding capacity of recombinant
proteins containing the K291A and/or K295A mutations was reduced on Huh7 cells, but not on C6/36 cells. The double-arginine mutant K291R/K295R/K305A/K307A/K310A was observed to bind with significantly higher affinity compared with the corresponding alanine mutant K291A/K295A/K305A/K307A/K310A on Huh7 cells, suggesting the recovery of charge-dependent interactions (Fig. 4, dashed line). No observable binding difference was detected for any MBP–DIII constructs on C6/36 cells.

**Antiviral activity of MBP–DIII proteins**

Recombinant DIII has previously been shown to block both virus binding and E-mediated fusion in a number of flaviviruses (Chu et al., 2005; Hung et al., 2004; Liao & Kielian, 2005). To assess the relative ability of our MBP–DIII constructs to prevent virus entry, we performed infection in their presence and quantified virus infection by measuring viral non-structural protein 1 (NS1) expression in an in-cell Western assay. Antiviral activity was assessed in both Huh7 and C6/36 cell lines and is presented as the percentage reduction relative to an untreated control infection for each cell line (Fig. 5). MBP–DIII (WT) was determined to have an IC50 of 10 μM in insect cells and 13 μM in human cells. No effect was observed using MBP alone, which confirmed that the antiviral activity was due to DIII alone. Inhibition mediated by recombinant proteins containing the K291A and/or K295A mutations was reduced on Huh7 cells, but not on C6/36 cells. The double arginine mutant, K291R/K295R/K305A/K307A/K310A, inhibited virus infectivity at a significantly lower concentration (IC50 = 12 μM) than the corresponding alanine mutant K291A/K295A/K305A/K307A/K310A (100 μM) on Huh7 cells (Fig. 4, dashed line). No observable binding differences were detected for any MBP–DIII constructs on C6/36 cells.

**DISCUSSION**

GAG interactions are thought to play a key role in the initial binding of flaviviruses to the host cell (Chen et al., 1997; Germi et al., 2002; Hung et al., 1999; Kroschewski et al., 2003; Su et al., 2001). The E protein is proposed to mediate this interaction via surface-exposed charged residues (Chen et al., 1997), with sequence analysis
identifying two highly conserved putative GAG-binding regions that are both within DIII. We developed a GAG ELISA to test relative binding affinities of recombinant DIII constructs using a BSA–heparin conjugate as a substrate in a four-level ELISA format. Given that binding is based on charged interactions, it was not surprising that, during the process of assay optimization, we observed a pH dependence of binding for our recombinant MBP–DIII construct (Fig. 1a). In aqueous solution at higher pH, the -NH$_3^+$ at the end of lysine side chains would lose a hydrogen ion to generate a residue with a neutral charge, thereby reducing its interaction with the negatively charged GAG. The loss of binding at pH values below 6 may reflect protonation of histidine residues within DIII and the subsequent partial refolding of this domain, as is proposed to occur during endocytosis of the virus (Fritz et al., 2008; Kampmann et al., 2006; Mueller et al., 2008). This pH-induced protonation is thought to release E from its metastable dimeric form to a stable trimeric form, thereby driving fusion of the viral and host cellular membranes (Fritz et al., 2008; Kampmann et al., 2006; Mueller et al., 2008). As our primary focus was on receptor-binding events at the cell surface, we continued our investigations at the more relevant pH of 7.

Under these optimized ELISA conditions, we confirmed that the interaction was due to DIII alone and not the
MBP-fusion partner, firstly by demonstrating no direct binding of MBP to the immobilized BSA–heparin (Fig. 2b) and secondly in a competitive assay using soluble MBP and DIII (Fig. 2c). Binding of MBP–DIII was only inhibited using DIII, indicating that the GAG-binding property of the recombinant fusion proteins could be attributed solely to DIII. The competition was shown to be approximately equimolar, demonstrating that the relative avidity of the two constructs was probably similar and that any oligomeric interactions were not being inhibited sterically by the presence of the MBP-fusion partner. This is an often-overlooked aspect of recombinant protein–ligand interactions, especially in the case of highly multimeric virions, as oligomeric superstructures can contribute significantly to the receptor interaction, and binding motifs spanning multiple domains may be present on the virion but not in the recombinant protein. One such region of interest is the fivefold axis of the flavivirus virion, where DIII forms a pentameric ring (Fig. 1c). This arrangement has been proposed to allow the virus to interact with the primary receptor (Zhang et al., 2004). This would also fit with the observed binding site of DC-SIGN, which has been shown not to block access to this area in the presence of saturating levels of the carbohydrate-binding domain of DC-SIGN (Pokidysheva et al., 2006). Also, it should be noted that residues of GAG-binding region 1 lie in close proximity to each of the pentameric domains (Fig. 1c) and may therefore contribute to interactions with longer GAG chains, as has been observed experimentally (Chen et al., 1997). Whilst these multimeric interactions are outside the scope of the present study, further future investigation is warranted.

Having shown that GAG binding was DIII-specific and not due to the MBP-fusion partner, we then went on to...
determine the relative importance of each conserved lysine residue within the two recognized binding motifs by site-directed mutagenesis and deletion analysis. No alteration in GAG-binding affinity was observed for the changes within region 2 (Fig. 3a), so we focused upon region 1 for the remainder of the study. After identifying the importance of this motif, we sought to elucidate the relative importance of each targeted lysine residue and any synergistic effects by sequential mutagenesis. These studies revealed that recombinant proteins containing mutations of either K291 or K295 to alanine reduced GAG binding in the context of our GAG ELISA (Fig. 3). Recombinant proteins containing both of these mutations demonstrated an even greater reduction in binding (Fig. 3), suggesting that both residues are required to fully engage GAG side chains. We went on to confirm this result by conservative mutation to arginine of both these residues, which rescued the WT-binding phenotype even in the presence of alanine mutations to the remaining three lysine residues. This finding indicates that the positive charge of these two basic residues is critical for the GAG–DIII interaction, presumably via direct electrostatic interactions with the negative sulphate group within the carbohydrate ligand.

To determine the biological relevance of the GAG ELISA data, we subsequently investigated the binding and antiviral properties of our recombinant proteins within a cellular context. Using a fixed-cell-based binding assay, we examined the relative binding affinities of our recombinant proteins on both mammalian and insect cells. We chose to look at cell types from both hosts, as it has previously been shown that GAG interaction is only of importance with respect to mammalian cell entry of DENV (Hung et al., 2004; Thaisomboonsuk et al., 2005). Therefore, changes to amino acids critical to GAG interactions should only affect mammalian cell binding. This hypothesis also remains valid when using DIII as an entry inhibitor, as DIII binding to GAG is expected to

Fig. 5. Antiviral activity of MBP–DIII. Mutants bearing changes in critical residues identified in the GAG ELISA were examined. Cells were infected with DENV-2 at an m.o.i. of 2×10⁻⁵ in the presence of varying concentrations of recombinant proteins and productive virus infection was quantified 48 h later by detection of the viral protein NS1 by in-cell Western assay. Log₁₀IC₅₀ values were obtained by fitting a sigmoidal dose–response curve. Inhibition of DENV entry into the mammalian cell line Huh7 was reduced by alanine mutations to either K291 or K295 alone (light grey circle) or in combination (grey square). WT-like antiviral properties were observed for the recombinant containing the conservative mutations K291R and K295R in combination with K305A, K307A and K310A (dashed line/fill). No significant difference in antiviral effect was discernible between the mutants and WT MBP–DIII on C6/36 cells. MBP was shown to not have an effect on virus production for either cell line at any concentration tested (diamonds).
compete with whole virus in the mammalian context, but not during insect cell infection.

Indeed, when the cellular affinity of our recombinant proteins was assessed, binding was only altered for mutants containing changes to either K291 or K295 and this effect was only observed on Huh7 cells (Fig. 4). As we observed equal binding between all of the recombinant proteins tested on C6/36 cells, we can conclude that DIII-mediated DENV binding to mosquito cells is independent of the putative GAG-binding motif in region 1. This is in agreement with the finding of Hung et al. (2004), who demonstrated that the exposed FG loop (residues 380–389) of DIII mediates mosquito cell interactions. However, this does not rule out a role for regions within DI and DII, which could enhance the binding of DIII to the primary mosquito receptor. In addition to the observation of a reduction in binding capacity for recombinant proteins containing alanine mutations to K291 or K295 on Huh7 cells, we also observed that conservative mutation of these two residues to arginine ameliorated the reduction in binding, even in the context of alanine mutations to the remaining lysine residues (Fig. 4a). These results were mirrored in our investigations into the antiviral properties of DIII. Here again we observed a reduced ability of recombinant proteins containing changes to K291 or K295 to inhibit virus entry in Huh7 cells, but not C6/36 cells. This probably reflects the ability for DIII to compete for binding to GAG molecules in addition to the primary receptor on mammalian cell lines, while only inhibiting cell entry in insect cells through direct competition with the primary receptor or inhibition of the fusion process, as has been proposed previously (Liao & Kielland, 2005).

An important role for K291 and K295 in virus replication is supported by sequence analysis of a large number of low-passage clinical isolates (sequences retrieved from http://www.broadinstitute.org/), which reveals a high level of conservation (>99%) for these residues, while lysine residues within the A strand were not as well-conserved (K305 30%, K307 75%, K310 99%). It is also worth noting that alanine mutations to K305, K307 and K310 caused similar behaviour to WT in all three assays, despite previous work suggesting a role for residues 306–314 of the A strand in GAG binding (Thullier et al., 2001). That study mapped a neutralizing epitope to this region and demonstrated that the neutralizing antibody used also blocks DIII binding to heparin sulphate. As this study used an alternative DIII construct spanning residues 290–400, it is missing the critical residues identified in this investigation and therefore a direct comparison cannot be made. Interestingly, a recent nuclear magnetic resonance-based study has also identified a similar dependence on GAG region 1 of JEV DIII through lysine residues with the DI–III flexible hinge, but not lysine residues within the A strand (Chen et al., 2010). However, these authors did observe GAG interactions with Y301 along with other residues within DIII, so a more elaborate recognition motif involving hydrophobic interactions in addition to electrostatic interactions is likely to mediate GAG–E binding. This would be in agreement with our finding that alanine mutations to all lysine residues within region 1 do not fully abrogate GAG binding. Additional mutagenesis and structural studies will be needed to clarify the details of the full binding motif.

In this study, we have prepared a range of recombinant proteins in an effort to determine the residues critical for DIII–GAG interactions. Moreover, we have established a high-throughput ELISA-based method to determine the heparin-binding properties of our DIII constructs that could be applied to other virus–host interaction studies. Using this technique along with mutagenesis of our recombinant–expressed DIII, we identified two key residues, K291 and K295, involved in heparin binding. The WT binding phenotype can be recovered by conservative mutation of these residues to the positively charged arginine, indicating that the interaction is likely to be electrostatic in nature. Furthermore, we confirmed the biological relevance of these residues through two different cell-based assays: an on-cell Western binding assay to determine the binding affinities of our constructs to cell-associated GAGs and an antiviral assay to determine the relative importance of these residues in live virus–cell interactions. Both assays demonstrated the importance of residues K291 and K295 in a human cell line but not in an insect cell line, supporting the hypothesis that this region is an important GAG-binding determinant in mammalian cell entry only. Due to the high conservation of these positively charged residues (Fig. 1a), it is likely that the importance of this GAG-binding motif is shared among the different flaviviruses. These findings further contribute to our understanding of the molecular basis of flavivirus E–GAG interactions.

METHODS

Cloning and mutagenesis. Primer pair 01 and 02 (Supplementary Table S1, available in JGV Online), incorporating BanHI and HindIII restriction endonuclease sites and a C-terminal histidine tag, were used to amplify DIII residues 290–394 of the DENV-2 (ET300) E protein (GenBank accession no. AB021760) by RT-PCR. The amplified PCR products were subcloned into the pMal-C2X vector (NEB) using BanHI and HindIII restriction endonucleases. Site-directed mutations and deletions in DIII were generated by amplifying the entire pMal-DIII plasmid with PCR using Phusion polymerase (Finzymes) together with a pair of partially overlapping primers incorporating the required mutations (Supplementary Table S1). WT MBP–DIII fusion protein was generated by insertion of a stop codon using primer pair 03 and 04 before the histidine tag-encoding sequence. Single alanine mutations were obtained using primers 05–14, and deletion of K394 or both K394/K395 was performed using primers 15–19 with WT plasmid as the template. Multiple mutants were generated via sequential mutagenesis using appropriate primer pairs, which included the additional primers 19 and 20 due to the close proximity between lysine residues 305, 307 and 310. The double-arginine mutant, K291R/K295R, was generated using primer pair 21 with the mutant K291A/K295A as the template. The entire ORF of MBP–DIII was sequence confirmed for each construct.

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Recombinant protein expression and purification. Plasmids encoding the recombinant WT and mutant proteins were transformed into Escherichia coli strain BL21(DE3), and transformants were cultured overnight at 37 °C in 5 ml Luria–Bertani broth (LB) medium supplemented with ampicillin (100 µg ml⁻¹) and shaken at 220 r.p.m. These cultures were then used to inoculate 500 ml LB medium with ampicillin in 2 l flasks. Protein expression was induced with the addition of 300 µM IPTG at an OD₆₀₀ of 0.6. After 3.5 h expression at room temperature and shaking at 180 r.p.m., cell suspensions were centrifuged and cell pellets were lysed via sonication. Insoluble products were subsequently removed by centrifugation at 10000 g and supernatant from the centrifugation was applied to an amylose resin column at 4 °C according to the protocols provided by the manufacturer (NEB). Elutions were pooled and buffer-exchanged with PBS using a spin concentrator (Millipore). The concentration of recombinant protein preparations was confirmed by BCA assay (Pierce).

Soluble DIII was obtained by FXa protease digestion of the purified MBP–DIII constructs for 24 h at 37 °C and at a 1:200 protease:protein ratio. Cleaved MBP was separated from DIII by anion-exchange chromatography using a Mono-Q column (GE Healthcare).

GAG ELISA. Heparin (5 mg; Sigma H4784) in 400 µl dH₂O was added to 5 mg N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (Sigma) in 500 µl of EtOH and incubated at room temperature for 1 h. Ten milligrams of BSA (Sigma–Aldrich) in 500 µl dH₂O was then added and incubated at 4 °C for 18–24 h. Unconjugated heparin was removed by centrifugation at 3000 g using a spin concentrator (cut-off molecular mass, 50 000 Da). ELISA plates (Nunc Maxisorp) were coated with 50 µl BSA–GAG (5 µg ml⁻¹) per well in a carbonate buffer (29 mM Na₂CO₃, 71 mM NaHCO₃) pH 9.6, overnight at 4 °C. Plates were blocked with 200 µl 5 % skimmed milk powder (SMP; Diploma) per well in PBS with 0.05 % Tween-20 (PBS-T) and then recombinant proteins were added for 1 h at 37 °C. Plates were washed three times with PBS-T and incubated with rabbit anti-MBP (New England Biolabs) for 1 h at 37 °C. Plates were washed three times with PBS-T and then probed with anti-rabbit–HRP conjugate as above. Plates were again washed and 3,3′,5,5′-tetramethylbenzidine (TMB; ELISA Systems) substrate was added for 5 min. Colour reaction was arrested with 2 M H₂SO₄ and plates were read at 450 nm.

Cell-binding assay. Huh7 and C6/36 cells were seeded into 96-well microtitre plates and grown until confluent. When fully confluent, plates were washed with PBS and fixed with 4 % para-formaldehyde for 20 min at room temperature. Blocking solution (5 % SMP in PBS) was added and incubated for 1 h at 37 °C. Plates were washed and probed with protein constructs at differing dilutions depending on the experiment for 1 h at 37 °C and subsequently washed three times for 5 min with PBS. Plates were then probed using a rabbit anti-MBP antibody for 1 h at 37 °C, and washed as above. IRdye800-conjugated goat anti-rabbit IgG (0.2 mg per well; LI-COR Biosciences) was added and incubated at 37 °C in the dark for 1 h. The plates were washed as described above and then briefly dried in the dark. Microtitre plates were scanned and analysed using the LI-COR Biosciences Odyssey Infrared Imaging System. Binding constants were derived from saturation binding curves.

Antiviral assay. The antiviral effect of recombinant DIII was determined in microtitre plates and assessed using an in-cell Western protocol. Cells were seeded in a 96-well plate and incubated until 90 % confluent. Cells were subsequently washed with PBS and virus and protein were separately diluted in serum-free (SF) medium. Cells were then co-incubated with protein at appropriate dilutions and DENV at an m.o.i. of 0.002. Mock-infected cells were incubated with SF medium. After 2 h, the medium was overlaid with M199 medium (Invitrogen) supplemented with FBS and carboxymethyl cellulose to give a final concentration of 1.5 %. After 48 h, cells were washed with PBS, fixed with 4 % para-formaldehyde for 20 min at RT and permeabilized with two 5 min washes in PBS containing 0.1% Triton X-100 (Sigma). Blocking solution (5 % SMP in PBS-T) was added and plates were incubated for 1 h at 37 °C. Plates were washed and probed using a rabbit anti-NS1 polyclonal serum (1:1000 in blocking solution; Invitrogen) and produced in house) for 1 h at 37 °C. The monolayer was then washed three times for 5 min with PBS-T. Alexa Fluor 680-conjugated goat anti-rabbit IgG (0.2 mg per well in blocking solution; Invitrogen) was added and incubated at 37 °C in the dark for 1 h. The plates were washed as described above, briefly dried in the dark and then scanned and analysed using the LI-COR Biosciences Odyssey Infrared Imaging System. IC₅₀ values were determined from dose–response curves.

ACKNOWLEDGEMENTS

We thank the National Health and Medical Research Council (NHMRC) of Australia for research support and the University of Queensland for a Postgraduate Research Scholarship for D.W. B.K. is an NHMRC Research Fellow.

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