Major Article

The role of heparan sulfate proteoglycans as an attachment factor for rabies virus entry and infection

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37 **Brief summary**

38 This study demonstrated that cellular heparan sulfate functions as the attachment host
39 factor for rabies virus infection through the interaction with the viral glycoprotein.

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Footnotes

Potential conflicts of interest. All authors: No reported conflicts.

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Abstract

Rabies virus (RABV) is the causative agent of fatal neurological disease. Cellular attachment is the initial and essential step for viral infections. Although extensive studies have demonstrated that RABV utilizes various target cell molecules to mediate infection, no specific molecule has been identified as an attachment factor for RABV infection. Here we demonstrate that cellular heparan sulfate (HS) supports RABV adhesion and subsequent entry into target cells. Enzymatic removal of HS reduced the cellular susceptibility to RABV infection and heparin, a highly sulfated form of HS, blocked viral adhesion and infection. The direct binding between RABV glycoprotein and heparin was demonstrated and this interaction was shown to require HS *N*- and 6-*O*-sulfation. We also revealed that basic amino acids in the ectodomain of RABV glycoprotein serve as major determinants for the RABV-HS interaction. Collectively, our study highlights a previously undescribed role of HS as an attachment factor for RABV infection.

Keywords

rabies virus; heparan sulfate; viral attachment; viral glycoprotein; host-virus interaction

Background

Rabies virus (RABV), genus *Lyssavirus*, family *Rhabdoviridae*, is the causative agent of rabies, a fatal neurological disease in mammals, including humans. For establishment of RABV infection, the virion attaches to target cells and binds to a RABV-specific receptor molecule, followed by cellular internalization by endocytosis [1, 2]. RABV utilizes several host membrane molecules as entry receptors, including the nicotinic acetylcholine receptor, neural cell adhesion molecule (NCAM) and nerve growth factor receptor, p75NTR [3-5]. While characterization of the interaction of RABV with these receptors has facilitated our understanding of RABV infection, the mechanism of cellular attachment and entry of RABV remains to be elucidated.

As for other viruses, cellular attachment is an initial step in RABV infection. Viral attachment factors are host molecules which facilitate viral adhesion on the cell surface and enhance infection, but do not trigger endocytic entry and membrane fusion of viruses. The glycoprotein (G) of RABV, the only transmembrane protein of RABV, binds to the entry receptors and plays critical roles during RABV entry [1, 2]. RABV G also binds various cell membrane components such as membranous proteins, gangliosides and lipids [6-9]. These studies suggested the existence of the attachment factor(s) which contributes to efficient adhesion of RABV; however, the molecules that serve as attachment factors for RABV infection are unidentified and the mechanism of cellular attachment of RABV remains unclear.

Heparan sulfate (HS), a glycosaminoglycan with a disaccharide repeating unit of glucosamine and uronic acid, is abundant on cellular surfaces as a component of HS proteoglycans. HS is involved in various biological events through interactions with cytokines, growth factors, proteases and membrane proteins [10]. Previous work has shown

that HS also binds certain viruses and mediates target cell infection [11-21]. Soluble forms of HS and heparin, a highly sulfated HS analog, can neutralize infection with viruses that utilize HS for cellular attachment by competitive inhibition for binding of cellular HS to the viruses [12-21]. HS and heparin have also been shown to inhibit RABV entry; however, this observation has been explained by the assumption that HS antagonizes the binding of RABV to the entry receptor NCAM, as HS also binds to NCAM [4]. Thus, the tripartite relationship between RABV, HS and NCAM is still unclear. In this study, we investigated the role of cellular HS on RABV infection and examined the interaction between HS and RABV.

Methods

Cells and viruses

SK-N-SH and NA cells were maintained in MEM with 10% fetal bovine serum (FBS). A549 cells were maintained in DMEM with 10% FBS. Huh-7 cells were maintained in RPMI-1640 with 10% FBS. The master stock of the RABV challenge virus standard (CVS) strain was prepared by intracranial infection of suckling mice. The master stock of RABV high egg passage Flury (HEP) strain was propagated in NA cells. Working stocks of the RABV strains were propagated in NA cells once and titrated by focus forming assays on NA cells as described previously [22].

Quantification of infectivity

SK-N-SH and Huh-7 cells were infected with RABV at a multiplicity of infection (MOI) of 5. A549 cells were infected with RABV at a MOI of 25. The indicated MOIs enable to evaluate for the effect of treatments. Following incubation for 1 h, cells were washed with PBS and cultured for 18 h. Cells were fixed with 3.7% buffered formaldehyde, permeabilized with PBS/0.2% Triton X-100 and then stained with FITC-conjugated anti-RABV monoclonal antibody (800-092, Fujirebio Diagnostics) and Hoechst 33342 (Molecular Probes). Images were captured with an automated microscope IN Cell Analyzer 2000 (GE Healthcare), and infection rates were calculated by counting total cells and RABV-positive cells.

Enzymatic removal of cellular HS

Cells were treated with heparinase III in serum-free DMEM at 37°C for 1 h. After PBS washing, cells were infected with RABV. Viral infectivity was quantified as described

144 above.

145

146 **Virus infection assay with HS**

147 For evaluation of the effect of viral treatment with HS, RABV was treated with a range of
148 dilutions of heparin (Wako), HS (Iduron), 2-O-desulfated heparin (Iduron), 6-O-desulfated
149 heparin (Iduron) or de-N-sulfated heparin (Sigma-Aldrich) for 1 h, then inoculated to cells.
150 Following incubation for 1 h, cells were washed with PBS and cultured for 18 h. For
151 evaluation of the effect of cellular treatment with HS or heparin, cells were treated with
152 heparin at 37°C for 1 h, thoroughly washed with PBS twice and infected with RABV. Viral
153 infectivity was quantified as described above.

154

155 **Pull-down assays**

156 RABV and heparin sepharose (Abcam) were incubated in 0.1 M phosphate buffer (PB, pH
157 7.0) with/without 300 μg/ml of heparin at 4°C for 1 h. Glutathione sepharose 4B (GE
158 Healthcare) was used as control. After PB washing, proteins were eluted and resolved by
159 SDS-PAGE, and detected by immunoblotting with anti-RABV N (3R7-5B12, HyTest).

160

161 **Preparation of a recombinant glycoprotein and surface plasmon resonance (SPR)**
162 **analysis**

163 A recombinant trimeric form of CVS-G (rG) was prepared by baculovirus expression
164 (Invitrogen). SPR measurements were performed using Biacore 3000 (GE Healthcare) as
165 described previously [20]. Further information is detailed in supplemental methods.

166

167 **Gene knockdown**

Three siRNAs targeting each gene (Silencer Select pre-designed siRNA, Ambion) were examined independently. Silencer Select negative control siRNA was used as a control. Cells were reverse transfected with 20 nM of siRNA using Lipofectamine RNAiMAX (Invitrogen) in 96-well plates. Following culture for 68 h, cells were infected with RABV. Viral infectivity was quantified as described above. Cell toxicities of the gene knockdowns were assessed with the CellTiter-Glo assay (Promega).

Pseudotyped-VSV infection assay

RABV *G* genes were cloned into the pCXSN [23] or pCI (Promega) vectors. HEP/CVS chimeric G-expressing plasmids were generated by exchanging partial regions of HEP-G with the homologous region of CVS-G using In-Fusion HD Cloning kit (Clontech). G mutants were generated by site-directed mutagenesis of plasmids. Replication-incompetent vesicular stomatitis virus (VSV) containing the green fluorescent protein (GFP) gene (VSVΔG*) was generated as described previously [24]. VSVΔG* pseudotyped with RABV G or G mutants were treated with/without 100 μg/ml of heparin at 37°C for 30 min and inoculated to SK-N-SH. Infectivity was calculated by counting nuclei stained with Hoechst 33342 and GFP-expressing cells with IN Cell Analyzer 2000 (GE Healthcare).

Statistical analysis

One-way analysis of variance (ANOVA) with Dunnett's test was used to determine statistical significance.

Results

Soluble HS neutralizes RABV infection in neuronal and non-neuronal cells

Employing a RABV CVS strain, we initially assessed the anti-RABV activity of soluble HS upon infection of neuronal SK-N-SH cells expressing NCAM (Figure 1A). Preincubation of RABV with soluble HS neutralized viral infectivity in a dose-dependent manner (Figure 1B) and heparin exhibited a stronger anti-RABV effect than HS (Figure 1C). Preincubation of SK-N-SH cells with HS or heparin, followed by PBS washing to remove unbound HS and heparin, showed no effect on RABV infectivity (Figure 1D and 1E). These results suggested that the neutralization activities of soluble HS and heparin on RABV infectivity are related to an interaction with RABV rather than NCAM on cells.

To further clarify the anti-RABV activity of soluble HS and heparin, we performed experiments employing NCAM-negative, non-neuronal Huh-7 cells (Figure 1A). As observed in SK-N-SH cells, preincubation of RABV CVS with either HS or heparin neutralized viral infectivity in a dose-dependent manner and heparin had a strong anti-RABV effect (Figure 1F and 1G). In addition, preincubation of Huh-7 cells with HS or heparin did not decrease RABV infectivity (Figure 1H and 1I). Comparable results were also obtained in experiments using another non-neuronal cells, A549 (Figure S1A-E). Taken together, these findings indicate that exogenous soluble HS or heparin neutralizes RABV infection independent of cellular NCAM.

Cellular HS is required for RABV infection of neuronal and non-neuronal cells

To further examine the role of cellular HS proteoglycans upon RABV infection, we inoculated SK-N-SH cells with RABV CVS after enzymatic depletion of cellular HS. Treatment with heparinase III reduced surface HS of SK-N-SH cells (Figure 2A) and

reduced the cellular susceptibility to RABV infection in a dose-dependent manner (Figure 2B and 2C). Similar to SK-N-SH, treatment with heparinase III inhibited RABV infection to both Huh-7 (Figure 2D) and A549 cells (Figure S1F). These results indicate that cellular HS is required for the efficient RABV infection of both neuronal and non-neuronal cells.

RABV and its glycoprotein directly bind to heparin

As preincubation of RABV with heparin, but not cells, inhibited viral infectivity, we assessed whether there was a physical interaction between heparin and RABV. Pull-down experiments with RABV CVS virions and heparin-conjugated sepharose beads were performed. RABV specifically bound to heparin-conjugated beads and this interaction was reduced by addition of heparin to the mixture of RABV and heparin-conjugated beads (Figure 3A). To elucidate the anti-RABV mechanism of heparin, we examined the cellular attachment of RABV in the presence of heparin. After incubation of RABV virions with cells at 4°C, attached virions were detected by flow cytometry. Pretreatment of virions with heparin markedly reduced viral attachment with cell surface (Figure 3B). These results indicate that heparin can directly bind to RABV virions, and that this interaction decreases viral attachment on the target cell surface.

Among the five structural proteins of RABV, G is the major protein distributed on the viral envelope in a homotrimeric structure [25]. Therefore, we hypothesized that G is a possible ligand for HS. To analyze the interaction of G derived from RABV CVS (CVS-G) and HS, we generated a recombinant trimeric form of CVS-G (rG) containing the ectodomain of CVS-G and the foldon trimerization domain [26]. We firstly confirmed that rG bound to cells (red line in Figure 4A) and the binding was inhibited in the presence of heparin (Figure 4A). The rG also neutralized the infectivity of RABV CVS, suggesting that

238 rG possesses a domain to attach to the cell surface competiting with RABV (Figure S2). We
239 analyzed rG binding to heparin by SPR assay. When rG was injected onto the
240 heparin-immobilized sensor chip, a binding response signal was observed and this
241 increased in a dose-dependent manner (Figure 4B). These results indicate that rG plays a
242 role as the ligand for heparin.
243
244 **Type *N*- and 6-*O*-sulfations on cellular HS are required for efficient RABV infection**
245 During HS biosynthesis, HS chains are modified by a series of sulfations: *N*-, 2-*O*-,
246 3-*O*- and 6-*O*-sulfations. The sulfation pattern of HS is dependent on cell type and modifies
247 ligand-binding properties [10]. To analyze the contribution of these sulfate modifications on
248 interactions between HS and RABV, we examined the neutralizing activity of desulfated
249 heparins on RABV CVS infection. Heparins selectively desulfated in *N*-sulfation (DeN),
250 2-*O*-sulfation (De2O) and 6-*O*-sulfation (De6O) were employed. The viral neutralizing
251 activity of De2O-sulfated heparin was comparable to that of native heparin, whereas DeN-
252 and De6O-sulfated heparins had no effect on viral infection at the examined concentrations
253 (Figure 5A). Using desulfated heparins, we estimated the interaction between each of the
254 desulfated heparins and rG by competition binding assays using SPR. Addition of either
255 heparin or De2O-sulfated heparin decreased the binding of rG to heparin in a
256 dose-dependent manner, whereas DeN- or De6O-sulfated heparins showed less inhibitory
257 effect on the binding of rG to heparin (Figure 5B), indicating that heparin and
258 De2O-sulfated heparin, but not DeN-sulfated heparin and De6O-sulfated heparin,
259 efficiently interacted with rG and interfered with the binding of rG to heparin. These data
260 suggest that *N*- and 6-*O*-sulfations of heparin are required for the binding to rG and
261 contribute to the antiviral neutralizing activity for RABV infection.

262 We examined the effect of sulfate modifications of cellular HS on RABV infectivity.
263 Four isoforms of *N*-deacetylase/*N*-sulfotransferase (encoded by *NDST1-4*),
264 2-*O*-sulfotransferase (*HS2ST1*) and three isoforms of 6-*O*-sulfotransferase (*HS6ST1-3*) have
265 been identified as host enzymes catalyzing the reactions of *N*-, 2-*O*- and 6-*O*-sulfations in
266 cellular HS biosynthesis, respectively [10]. Endogenous expression of the eight genes in
267 SK-N-SH was confirmed by RT-qPCR (Figure S3). At least two siRNAs out of three tested
268 targeting *NDST1*, *NDST2*, *NDST3* and *HS6ST1* significantly decreased RABV CVS
269 infection to SK-N-SH cells (Figure 5C) with no cytotoxic effect (Figure S4). These results
270 indicate that *N*- and 6-*O*-sulfations of cellular HS are required for the efficient RABV
271 infection of target cells.
272
273 **Cellular HS has no role in infection with RABV HEP strain**
274 We examined the interaction of HS to another strain of RABV, HEP, an attenuated
275 virus strain [27]. The G of RABV HEP (HEP-G) shares 90.6% amino acid identity to
276 CVS-G (GenBank accession numbers AB085828 and LC325820). Surprisingly, unlike
277 CVS (Figure 1B and 1C), preincubation of HEP with either HS or heparin had no effect on
278 viral infectivity in SK-N-SH cells (Figure S5A and S5B). HEP infection was also resistant
279 to enzymatic degradation of cellular HS by treatment with heparinase III (Figure S5C) in
280 contrast to CVS infection (Figure 2C). These results indicate that the infection by RABV
281 HEP strain of target cells is independent of cellular HS.
282
283 **Identification of critical amino acid residues in CVS-G for the interaction with**
284 **heparin**
285 We examined the CVS-G region responsible for binding to HS using VSV pseudotyped

286 with chimeric G mutants of CVS and HEP. VSVΔG* is a replication-incompetent VSV
287 lacking the glycoprotein gene [24]. As reported [15], preincubation with heparin had no
288 effect on the infectivity of VSVΔG* pseudotyped with VSV G (VSV-G) (Figure 6A).
289 Furthermore, preincubation with heparin reduced the infectivity of VSVΔG* pseudotyped
290 with CVS-G, but not HEP-G (Figure 6A), consistent with results of neutralizing assays
291 with heparin using infectious RABV CVS and HEP (Figure 1C and S5B). We prepared a
292 series of VSVΔG* pseudotyped with chimeric mutants between HEP-G and CVS-G as
293 shown in Figure 6B. Preincubation with heparin reduced the infectivity of VSVΔG*
294 bearing HC(126-273), but not HC(1-125) and HC(274-407) (Figure 6C), indicating that the
295 region between amino acid positions 126 to 273 of CVS-G was responsible for the
296 recognition and neutralization by heparin. In addition, treatment with heparin neutralized
297 the infectivity of VSVΔG* bearing HC(126-223) and HC(182-273), but not HC(126-181),
298 HC(182-223) and HC(224-273) (Figure 6C), suggesting that multiple sites between CVS-G
299 positions 126-273 are involved in the heparin neutralization.
300 To determine residues of CVS-G targeted by heparin, we constructed a series of
301 HEP-G mutants with multiple reciprocal substitutions between positions 126-273 and
302 identified three candidates (Figure S6). Preincubation with heparin decreased the infectivity
303 of VSVΔG* pseudotyped with HEP-G mutants carrying multiple substitutions at positions
304 158, 186 and 248 [i.e. HEP-G(158K/186R), HEP-G(158K/248K), HEP-G(186R/248K) and
305 HEP-G(158K/186R/248K)], while VSVΔG* bearing single substitutions [i.e.
306 HEP-G(158K), HEP-G(186R) and HEP-G(248K)] were resistant to the treatment (Figure
307 6D). Conversely, we introduced multiple reciprocal substitutions at the same three positions
308 of CVS-G and confirmed that 158N, 186G and 248E substitutions confer additive
309 resistance to preincubation with heparin to CVS-G pseudotyped VSVΔG* (Figure S7A).

These results indicated that residues at positions 158, 186 and 248 define the differential susceptibility to preincubation with heparin in both CVS-G and HEP-G carrying viruses.

It has been reported that clusters of basic amino acids, such as lysine (K) or arginine (R), in viral proteins, mediate HS binding [18, 28, 29]. To examine the contribution of the basic residues 158, 186 and 248 of RABV G to heparin neutralization, we generated HEP-G mutants with basic or neutral amino acid substitutions at these positions. Preincubation with heparin reduced the infectivity of VSVΔG* pseudotyped with HEP-G mutants with basic amino acid substitutions (K or R), but not neutral amino acid substitutions (alanine, A) at 186 and 248 (Figure S7B). However, heparin neutralized VSVΔG* pseudotyped with HEP-G carrying either basic or neutral amino acid substitutions at 158 (Figure S7C). These results indicate that the basic residues at 186 and 248 influence RABV neutralization by heparin, but residue 158 affects RABV infectivity by another mechanism(s).

Sensitivity of RABV G to heparin treatment exhibits strain variation

In addition to CVS and HEP, the sensitivity of RABV G to heparin treatment was examined employing the G of ERA and Nishigahara: fixed strains and 1088: a street strain. Preincubation with heparin neutralized VSVΔG* pseudotyped with G of both Nishigahara and 1088, while the inhibitory effects were relatively weak compared with that to VSVΔG* bearing CVS-G (Figure S8A). In contrast, preincubation with heparin had no effect on the infectivity of VSVΔG* bearing G of ERA (Figure S8A). Multiple sequence alignment based on glycoproteins of RABV fixed strains (CVS, HEP, ERA, Nishigahara) and street strains (1088, 9147FRA, 8764THA) showed that K158 is conserved but R186 and K248 are not conserved between the glycoproteins of RABVs sensitive to heparin pretreatment

334 (i.e. CVS, Nishigahara and 1088) (Figure S8B). These results suggest that G of some
335 RABV strains recognize heparin but the molecular binding modes are different from that of
336 CVS.
337

Discussion

Previous studies implicated the presence of RABV attachment factors in the cellular membrane fraction, however, no specific molecule has been identified [2]. Here, we show that cellular HS supports the RABV CVS infection (Figure 2). We also have identified CVS-G as a ligand for HS and characterized the interaction between HS and CVS-G (Figure 4). Based on these results, we conclude that cellular HS functions as the first identified attachment host factor for RABV infection through the interaction with the viral glycoprotein. A previous study showed that the exogenous expression of RABV receptor confers susceptibility to RABV infection of non-permissive cells [4]. The ubiquitous expression of HS on cells permissive and non-permissive for RABV indicates that HS serves an attachment factor rather than entry receptor for RABV infection.

The interactions with HS and proteins are partially mediated by an electrostatic binding between negatively charged chains of HS and basic amino acids in the target proteins. In addition, some viral proteins require specific *N*- or *O*- sulfation for HS interaction [17, 30-34]. Indeed, we have demonstrated that RABV CVS requires *N*- and 6*O*-sulfations of HS, but not 2*O*-sulfation, for the interaction with HS and efficient infection to cells (Figure 5). These observations strongly support that RABV CVS certainly recognizes cellular HS and utilizes this for infection.

The antiviral effects of heparin on RABV infection has been considered to be related to competition for the binding to NCAM, a RABV receptor [4]. Our study proposes an alternative mechanism whereby heparin inhibits RABV cell surface attachment through the direct binding to RABV (Figure 3B). Notably, the neutralization effect of preincubation of heparin with RABV CVS was much stronger than that of preincubation of heparin with cells, and the cell surface attachment of RABV CVS was decreased in the presence of

heparin (Figure 1C and 1E). These results indicate that the anti-RABV effect of heparin mainly depends on the attachment inhibition of RABV to cellular surface through its interaction with RABV. We also demonstrated that Huh-7 and A549 cells are deficient in NCAM expression but susceptible to CVS infection (Figure 1 and S1), indicating that the expression of NCAM is unnecessary for RABV infection of these cells. Importantly, the NCAM-independent antiviral effect of heparin was also clearly observed in these NCAM-negative cells (Figure 1G and S1C).

Reciprocal amino acid substitutions at positions 158, 186 and 248 switched the different susceptibility to heparin treatment between CVS-G and HEP-G (Figure 6D and S7A), suggesting these residues are responsible for the recognition by cellular HS. Other studies revealed that some viruses possess basic residues critical for the interaction with HS in their structural proteins [18, 28, 29]. Site-directed mutagenesis of RABV G demonstrated that the basic residues at 186 and 248 are key for the interaction of G to HS (Figure S7B). Conversely, the residue 158 seems to be involved in the HS interaction by a different mechanism(s) (Figure S7C). The asparagine (N) residue at 158 of HEP-G forms the consensus motif for N-linked glycosylation (N-x-S), which promotes RABV production [35]. The reciprocal substitution (158N) in CVS-G introduced a potential N-linked glycosylation site at the region (i.e. K-C-S to N-C-S). Therefore, one possible explanation is that N-linked glycosylation at position 158 interferes with the interaction of G to HS.

Heparin neutralized VSVΔG* pseudotyped with G of both fixed and street strains of RABV except for HEP and ERA (Figure 6 and S8A). Indeed, RABV HEP was resistant to preincubation with soluble HS and heparin, and infected cells independently of cellular HS (Figure S5). These results suggest that certain RABV strains utilize cellular HS as an attachment factor, however, others such as HEP and ERA are likely to utilize other

386 unknown molecules for cellular attachment. We largely employed the fixed strains of
387 RABV for this study. Further studies are needed to examine the interaction of HS with
388 other strains, and especially circulating street strains of RABV.

389 Previous studies demonstrated that the ability to bind HS influences the pathogenicity
390 of several viruses [12, 14, 36-41]. Field isolates of eastern equine encephalitis virus bind to
391 HS and that this interaction contributes to neurovirulence [14]. In addition, a single
392 mutation in E2 protein confers HS-binding property and increased neurovirulence of
393 Sindbis virus [36]. In contrast, HS-binding is often involved in virulence attenuation of cell
394 culture-adapted viruses [12, 37-41]. Notably, our heparin neutralization assays suggest that
395 pathogenic strains of RABV (i.e. CVS, Nishigahara, 1088) but not attenuated strains (HEP,
396 ERA), bind to HS (Figure S8A). The contribution of HS-binding property to the
397 pathogenicity of RABV requires further studies.

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500
501

Figure legends

Figure 1. Antiviral effect of exogenous HS and heparin on the infectivity of RABV CVS strain.

(A) Cellular surface expression level of NCAM was confirmed by flow cytometry using anti-NCAM antibody. Control mouse IgG2b antibody was used as a staining control. (B and C) RABV CVS was mixed with the indicated concentrations of HS (B) and heparin (C), and then inoculated to SK-N-SH cells. RABV-infected cells were identified by indirect immunofluorescence with anti-RABV N antibody and Hoechst 33342, and counted by imaging cytometry. (D and E) SK-N-SH cells were incubated with HS (D) or heparin (E) and repeatedly washed with PBS prior to the infection with RABV CVS. RABV-infected cells was analyzed as described above. (F-I) The same experiments (B-E) were performed using Huh-7 cells. The values in the graphs are shown as means ± S.D. of triplicates from a representative experiment. Statistical analyses were performed using one-way ANOVA with Dunnett's test. * $p < 0.01$, significantly different from the control.

Figure 2. Effect of enzymatic removal of cellular HS on the infectivity of RABV CVS.

(A) SK-N-SH cells were treated with the indicated concentrations of heparinase III and then analyzed by flow cytometry with anti-HS IgM antibody. Control mouse IgM was used as control antibody. (B and C) SK-N-SH cells treated with heparinase III were infected with RABV CVS. The cells were stained with anti-RABV N antibody (green) and Hoechst 33342 nuclear dye (blue). The infected cells were subjected to fluorescent microscopy analysis (B) and counted by imaging cytometry (C). Scale bars, 100 μm. (D) Huh-7 cells were pretreated with heparinase III and then infected with RABV CVS. Cells were stained and counted by imaging cytometry as described above. The values in the graphs are shown

as means ± S.D. of triplicates from a representative experiment. Statistical analyses were performed using one-way ANOVA with Dunnett's test. * $p < 0.01$, significantly different from the control.

Figure 3. Direct binding of heparin to RABV CVS.

(A) Control or heparin sepharose beads were incubated with RABV CVS in PBS. Heparin was used to compete the binding of RABV CVS to beads. The viruses binding to the beads were detected by immunoblot with anti-RABV N protein. (B) RABV CVS and detached cells were incubated in the absence or presence of heparin at 4°C. The attached viruses on the cell surface were detected by flow cytometry with anti-RABV G protein.

Figure 4. Direct binding of heparin to RABV glycoprotein G.

(A) Recombinant soluble CVS-G protein was incubated with SK-N-SH cells at 4°C. The binding of G protein was detected by flow cytometry with anti-RABV G protein. (B) Sensorgrams of the binding of recombinant CVS-G protein (rG) to heparin immobilized on a sensor chip by SPR analysis. RU, response units.

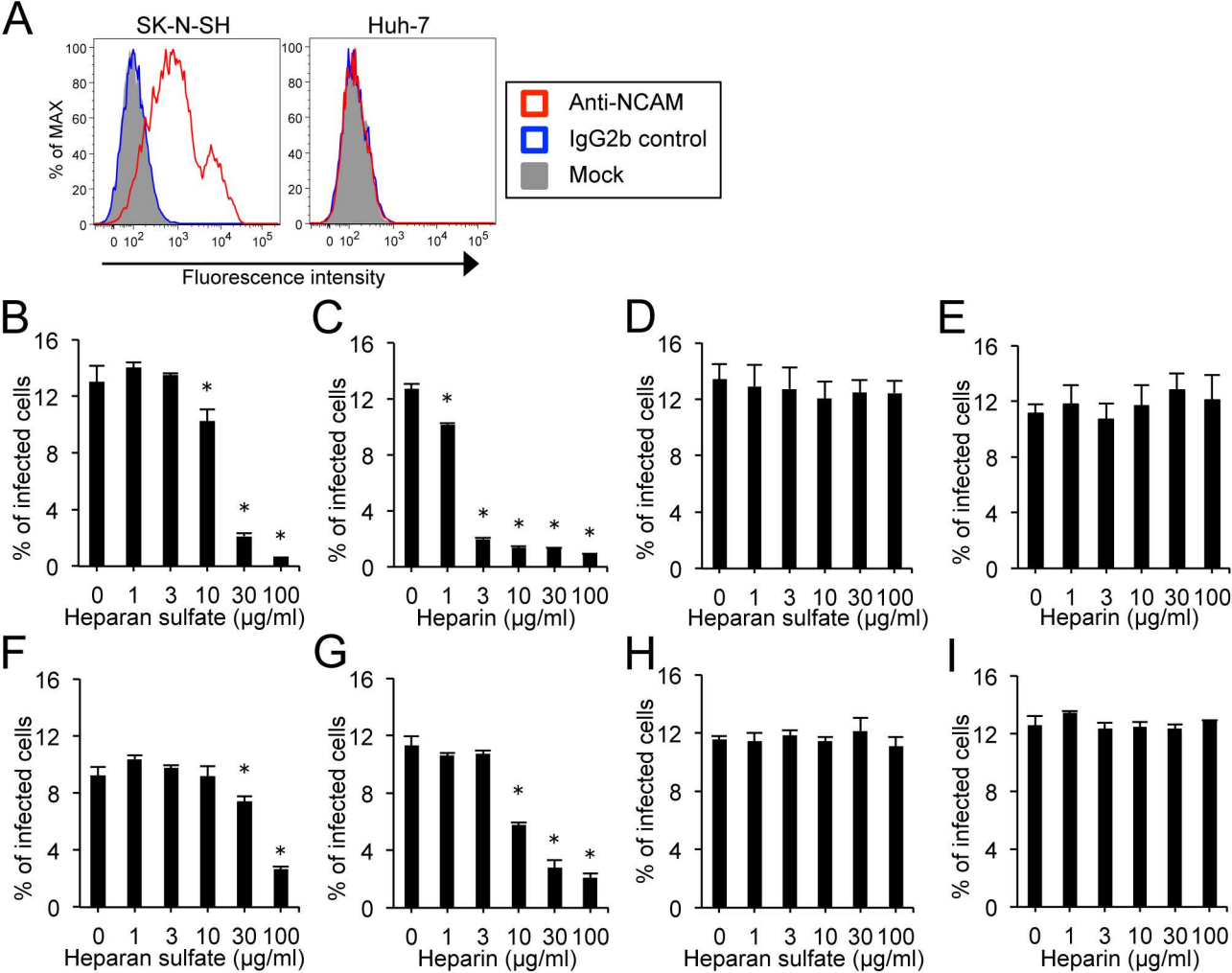
Figure 5. Contribution of sulfation types of heparin and HS for their interaction with RABV CVS.

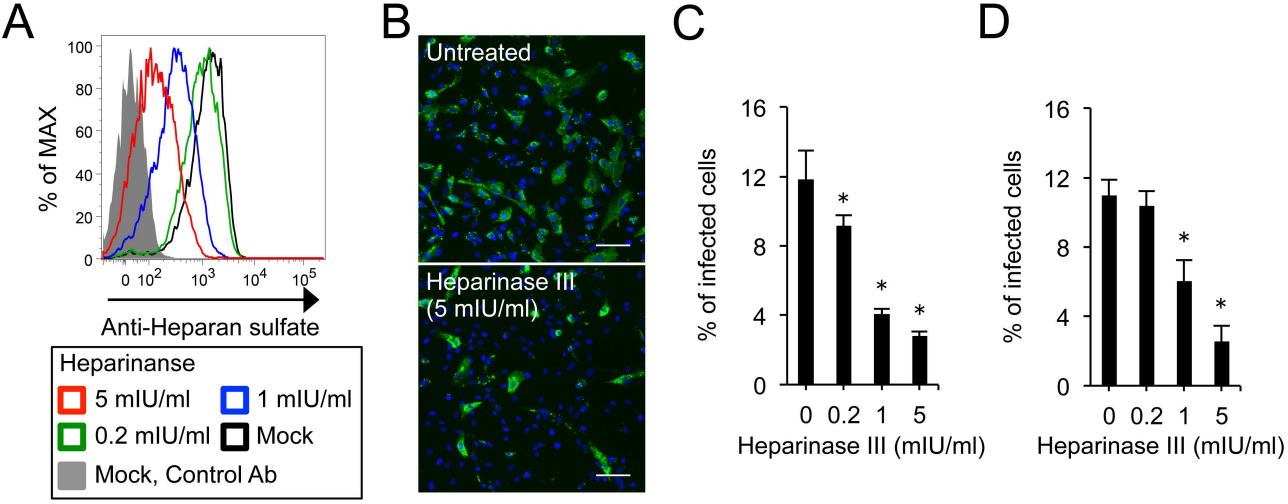
(A) RABV CVS was mixed with either heparin or the indicated forms of desulfated heparin, and then inoculated to SK-N-SH cells. Cells were stained with anti-RABV N antibody and Hoechst 33342, and counted by imaging cytometry. Relative infectivity was calculated by setting the infectivity of mock-treated RABV CVS to 100%. (B) Sensorgrams of the binding of recombinant CVS-G protein (rG) to heparin immobilized on a sensor chip, after

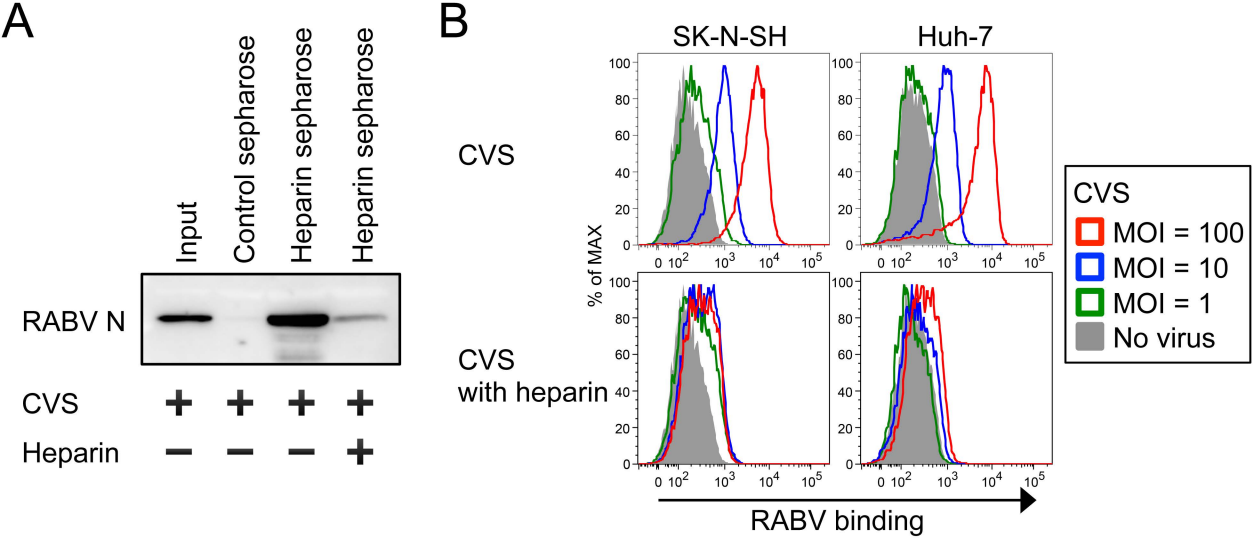
preincubation with heparin or the indicated forms of desulfated heparin. RU, response units. (C) RABV CVS was inoculated with SK-N-SH cells transfected with siRNAs targeting the indicated genes. Three siRNAs were assayed for each gene knockdown. Cells were analyzed by imaging cytometry and relative infectivity was calculated as described above. The values in the graphs are shown as means ± S.D. of triplicates from a representative experiment. Statistical analyses were performed using one-way ANOVA with Dunnett's test. * $p < 0.01$, significantly different from the control.

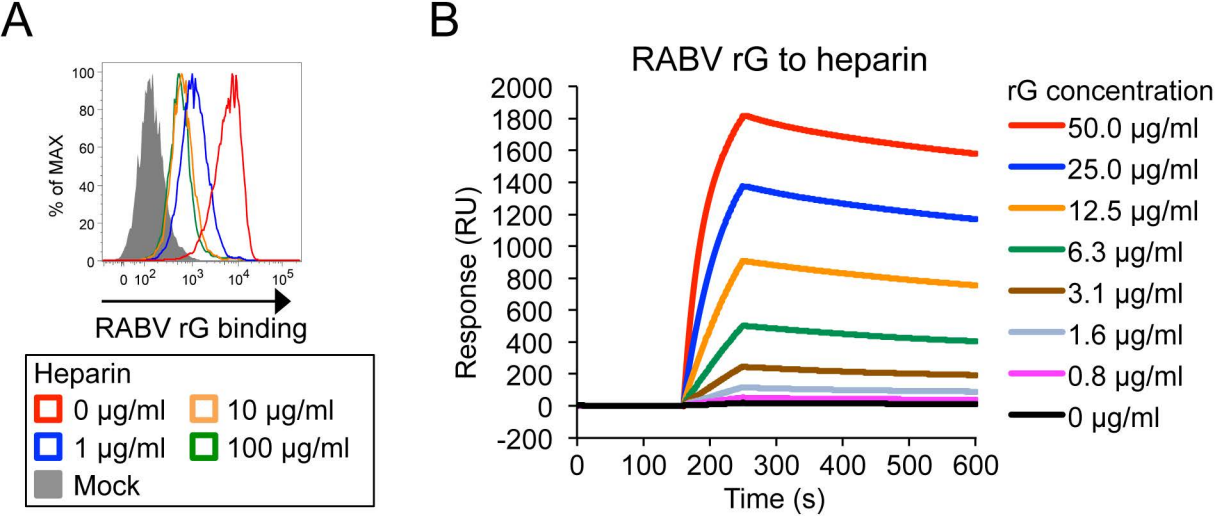
Figure 6. Effect of heparin treatment on the infectivity of pseudotyped VSVΔG*.

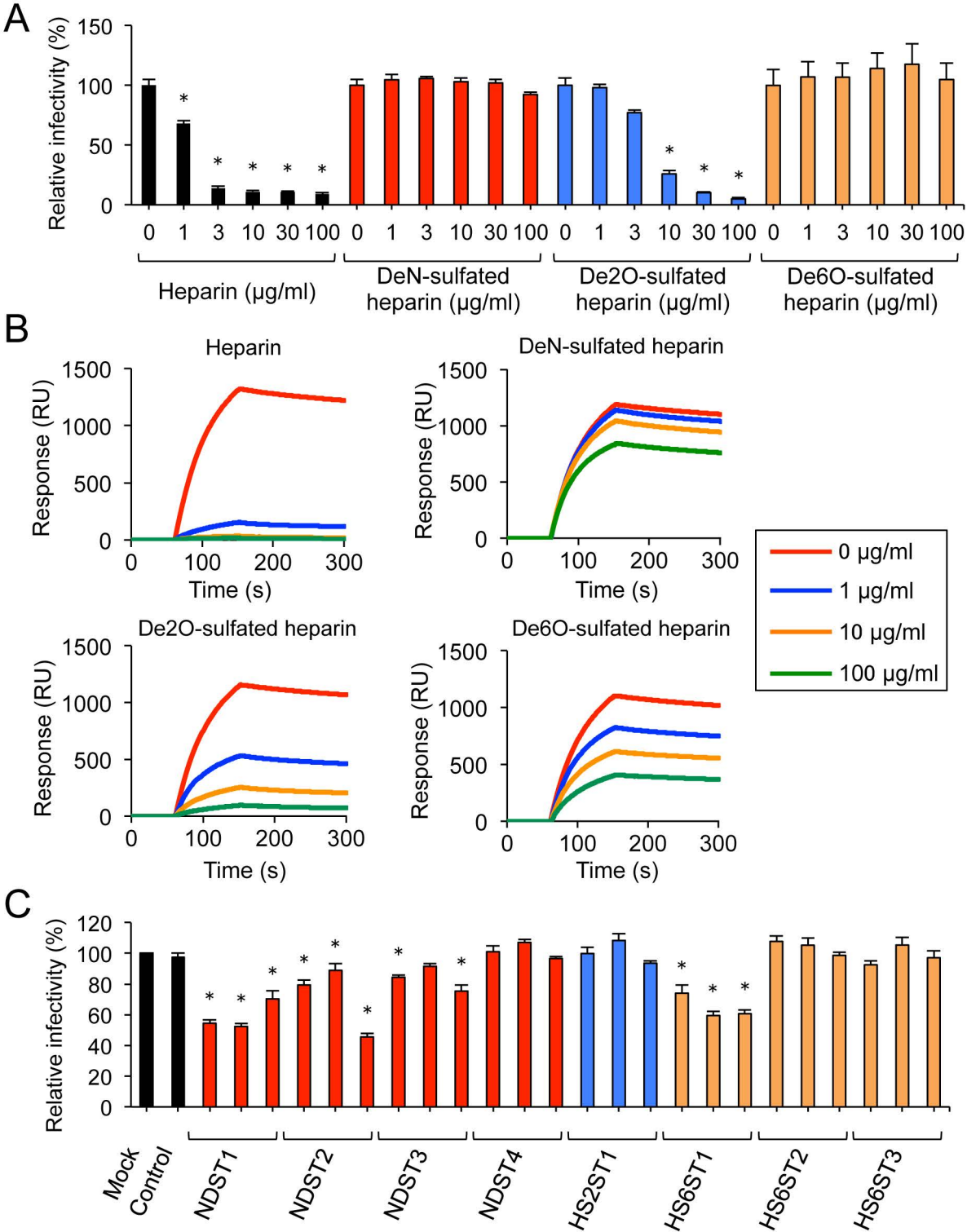
(A) VSVΔG* pseudotyped with G proteins of VSV (VSV-G), HEP (HEP-G) or CVS (CVS-G) were pretreated with 100 μg/ml of heparin and then inoculated to SK-N-SH cells. Infection with pseudotyped VSVΔG* was identified by GFP reporter expression. Cellular nuclei were stained with Hoechst 33342. The number of GFP-positive cells and total cells were counted by imaging cytometry. Relative infectivity was calculated as a percentage of untreated conditions. (B) Schematic representation of chimeras used in this study. The numbers indicate amino acid positions of mature G protein without the signal peptide. TM, transmembrane domain. (C and D) VSVΔG* pseudotyped with chimeric G (C) or point mutants of HEP-G (D) were pretreated with 100 μg/ml of heparin and then inoculated to SK-N-SH cells. Cells were analyzed by imaging cytometry and relative infectivity was calculated as described above. The values in the graphs are shown as means ± S.D. of triplicates from a representative experiment.

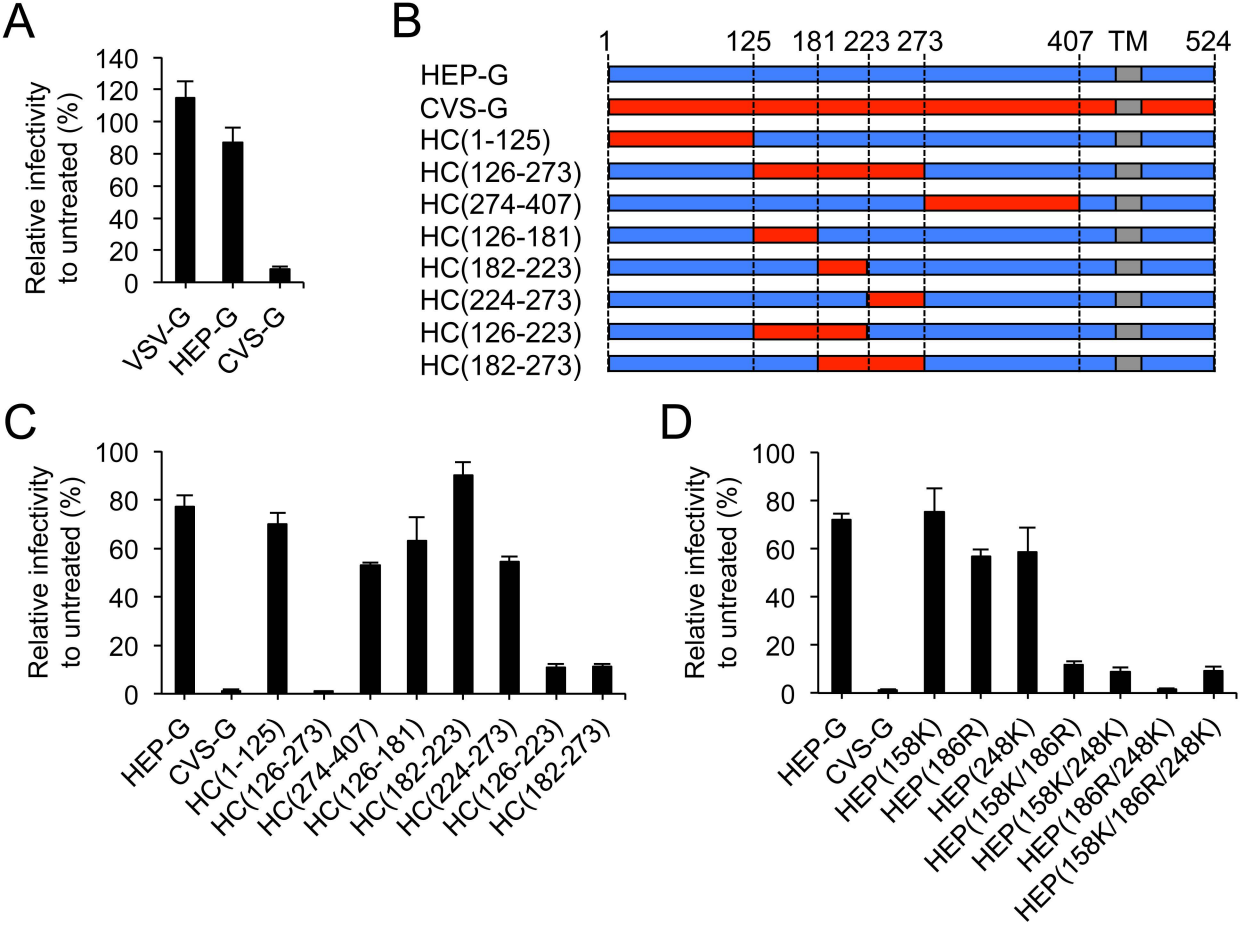

A
SK-N-SH
Huh-7
% of MAX
Fluorescence intensity
Anti-NCAM
IgG2b control
Mock
B
C
D
E
F
G
H
I
% of infected cells
Heparan sulfate (μg/ml)
Heparin (μg/ml)
0 1 3 10 30 100


A
% of MAX
100
80
60
40
20
0
0 10^2
10^3
10^4
10^5
Anti-Heparan sulfate
Heparinanse
5 mIU/ml
1 mIU/ml
0.2 mIU/ml
Mock
Mock, Control Ab
B
Untreated
Heparinase III
(5 mIU/ml)
C
% of infected cells
16
12
8
4
0
0 0.2 1 5
Heparinase III (mIU/ml)
D
% of infected cells
16
12
8
4
0
0 0.2 1 5
Heparinase III (mIU/ml)


A
Input
Control sepharose
Heparin sepharose
Heparin sepharose
RABV N
CVS + + + +
Heparin − − − +
B
SK-N-SH
Huh-7
CVS
CVS with heparin
% of MAX
RABV binding
CVS
MOI = 100
MOI = 10
MOI = 1
No virus


A
100
80
60
40
20
0
% of MAX
0 10^2 10^3 10^4 10^5
RABV rG binding
Heparin
0 µg/ml
10 µg/ml
1 µg/ml
100 µg/ml
Mock
B
RABV rG to heparin
2000
1800
1600
1400
1200
1000
800
600
400
200
0
-200
Response (RU)
0 100 200 300 400 500 600
Time (s)
rG concentration
50.0 µg/ml
25.0 µg/ml
12.5 µg/ml
6.3 µg/ml
3.1 µg/ml
1.6 µg/ml
0.8 µg/ml
0 µg/ml


A
Relative infectivity (%)
150
100
50
0
0 1 3 10 30 100 0 1 3 10 30 100 0 1 3 10 30 100 0 1 3 10 30 100
Heparin (μg/ml)
DeN-sulfated heparin (μg/ml)
De2O-sulfated heparin (μg/ml)
De6O-sulfated heparin (μg/ml)
B
Heparin
DeN-sulfated heparin
De2O-sulfated heparin
De6O-sulfated heparin
Response (RU)
1500
1000
500
0
0 100 200 300
Time (s)
0 μg/ml
1 μg/ml
10 μg/ml
100 μg/ml
C
Relative infectivity (%)
120
100
80
60
40
20
0
Mock Control
NDST1
NDST2
NDST3
NDST4
HS2ST1
HS6ST1
HS6ST2
HS6ST3


A
Relative infectivity to untreated (%)
140
120
100
80
60
40
20
0
VSV-G
HEP-G
CVS-G
B
1
125
181
223
273
407
TM
524
HEP-G
CVS-G
HC(1-125)
HC(126-273)
HC(274-407)
HC(126-181)
HC(182-223)
HC(224-273)
HC(126-223)
HC(182-273)
C
Relative infectivity to untreated (%)
100
80
60
40
20
0
HEP-G
CVS-G
HC(1-125)
HC(126-273)
HC(274-407)
HC(126-181)
HC(182-223)
HC(224-273)
HC(126-223)
HC(182-273)
D
Relative infectivity to untreated (%)
100
80
60
40
20
0
HEP-G
CVS-G
HEP(158K)
HEP(186R)
HEP(248K)
HEP(158K/186R)
HEP(158K/248K)
HEP(186R/248K)
HEP(158K/186R/248K)