109   cells was inhibited by anti-PSGL-1 monoclonal antibody (KPL1), indicating that

110   EV71-PB replicated in Jurkat cells in a PSGL-1-dependent manner. On the other hand,

111   EV71 replicated in nonleukocyte cells (such as RD cells) expressing little or no PSGL-1,

112   and the replication was not affected by KPL1. Therefore we conclude that EV71 does

113   not use PSGL-1 as the major cellular receptor on RD cells and other receptor(s),

114   including SCARB2 or annexin II, may be responsible for EV71 infection in

115   nonleukocyte cells expressing little or no PSGL-1.

116

117   Post-translational modifications of the N-terminal region of PSGL-1 contribute the

118   efficient binding to selectins and chemokines (Pouyani and Seed, 1995;Sako et al.,

119   1995;Wilkins et al., 1995;Liu et al., 1998;Hirata et al., 2004). In this region, there are a

120   potential *O*-glycosylation residue (T57) and three potential tyrosine sulfation sites (Y46,

121   Y48, and Y51). We demonstrated that tyrosine sulfation, not *O*-glycosylation, of the

122   N-terminal region of PSGL-1 facilitates its binding to EV71-PB and viral replication in

123   Jurkat T cells (Nishimura et al., 2010).

124

125   CVA16 is genetically and antigenically related to EV71 and is a major causative agent

126   of HFMD as well as EV71 (Oberste et al., 2004). The inoculation of L-PSGL-1.1 cells,

127   mouse L929 cells stably expressing human PSGL-1, with the prototype CVA16-G-10

128   strain induced faint cytopathic effects (CPE). The replication of CVA16-G-10 was

129   partially inhibited by KPL1 in L-PSGL-1.1 cells. This result indicated that the prototype

130   CVA16 strain may use human PSGL-1 and another unidentified receptor(s) to infect

131   L-PSGL-1.1 cells. CVA16-G-10 replication in Jurkat cells was not apparently inhibited

132   by KPL1 (Nishimura et al., 2009), but significantly inhibited by a sulfation inhibitor,

133   sodium chlorate (Nishimura et al., 2010). Therefore some sulfated molecules other than

134   PSGL-1 might be involved in the replication of CVA16 in Jurkat cells. CVA16-G-10

135   would use unidentified receptor(s) to infect Jurkat T cells (Nishimura et al., 2009;Patel

136 and Bergelson, 2009).

137

138 To investigate the PSGL-1-dependent replication phenotype of HEV-A, we tested the

139 replication of 10 prototype HEV-A strains in L-PSGL-1.1 cells in the presence or

140 absence of the anti-PSGL-1 mAb, KPL1 (Figure 1A). On day 6 post-inoculation, there

141 was no significant replication of CVA4, CVA5, CVA6, or CVA8 in L-bsd cells

142 (blasticidin-resistant control L929 cells) or L-PSGL-1.1 cells. Although higher viral

143 titers were found for CVA2 and CVA7 in L-PSGL-1.1 cells compared with those in

144 L-bsd cells, replication was not affected by KPL1. These results suggest that CVA2 and

145 CVA7 may infect to L-PSGL-1.1 cells in an alternative pathway via PSGL-1 or

146 glycosylated PSGL-1, without the interaction between EV71-PB and the N-terminal

147 region of PSGL-1 recognized by KPL1. We could not demonstrate any

148 PSGL-1-dependent replication of the CVA3, CVA10, CVA12, and CVA14 strains in

149 L-PSGL-1.1 cells, because they replicated even in PSGL-1 negative L-bsd cells as

150 previously reported for certain HEV-A field isolates (Nadkarni and Deshpande, 2003;

151 Yamayoshi et al., 2009).

152

153 CVA7 and CVA14 infection induced CPE in L-PSGL-1.1 cells, but not in L-bsd cells

154 (Table 1). On the other hand, CVA7 and CVA14 induced CPE in L-Empty cells

155 (puromycin-resistant control L929 cells) (Table 1) (Yamayoshi et al., 2009). The

156 difference in the CPE induction by some HEV-A strains might be due to the

157 maintenance or cultivation conditions of the mouse L929-derived cells regardless of the

158 receptor expression of PSGL-1 or SCARB2. Some strains of HEV-A are able to infect

159 mouse L929 cells regardless of expression of PSGL-1 or SCARB2 (Nadkarni and

160 Deshpande, 2003; Yamayoshi et al., 2009). It is therefore impossible to determine

161 receptor usage of HEV-A by simply investigating the susceptibility of L929 cells

162 expressing the putative cellular receptor. Receptor usage of HEV-A should be

163   determined carefully by showing several lines of evidence such as acquisition of

164   susceptibility by expressing a putative receptor in nonsusceptible cells, loss of

165   susceptibility by knocking down of the receptor in susceptible cells, and direct binding

166   of the virus to the receptor, etc.

167

168   L-PSGL-1.1 cells did not support PSGL-1-dependent replication of the HEV-B and

169   HEV-C strains (Figure 1B); however, the prototype EV70 strain (HEV-D) replicated in

170   L-PSGL-1.1 cells more efficiently than in L-bsd cells. Although EV70 replication was

171   not affected by KPL1 (Figure 1B), we cannot exclude the possibility that EV70 utilizes

172   α2,3-linked sialic acid, which could be a receptor for EV70 (Nokhbeh et al., 2005).

173

174   Recently we reported that that four out of five EV71-PB strains replicated poorly in

175   L-PSGL-1.1 cells (Miyamura et al., 2011). We found that EV71 variants, which were

176   propagated once in L-PSGL-1.1 cells, have several possible adaptive mutations,

177   including a putative amino acid determinant of the adaptive phenotype in L-PSGL-1.1

178   cells at VP2-149 (Miyamura et al., 2011). The results suggest that adaptive mutations,

179   along with a PB phenotype, may facilitate efficient PSGL-1-dependent replication of

180   the EV71 variants in L-PSGL-1.1 cells. It is possible that HEV-A strains other than

181   EV71 also require adaptive mutations for efficient replication in L-PSGL-1.1 cells.

182

183   **3. SCARB2**

184   Yamayoshi et al. (2009) identified SCARB2 (also known as lysosomal integral

185   membrane protein II, or CD36b like-2) as an EV71 receptor on RD cells, widely used

186   for isolation of EV71 from clinical specimens. They transfected EV71-nonsusceptible

187   L929 cells with the genomic DNA of RD cells and selected two cell clones that were

188   susceptible for EV71 infection. By a transcriptome analysis, SCARB2 was identified as

189   an EV71 receptor on RD cells.

190

191   SCARB2 is a heavily *N*-glycosylated type III transmembrane protein consists from 478

192   amino acids and belongs to the CD36 family of scavenger receptor proteins (Fujita et al.,

193   1992;Calvo et al., 1995). SCARB2 has a N-terminal transmembrane domain, a ~400

194   amino acid lumeral domain, C-terminal transmembrane domain, and a C-terminal

195   cytoplasmic tail of ~20 amino acids (Fujita et al., 1992). SCARB2 involves in an

196   enlargement of early endosomes and late endosomes/lysosomes and an impairment of

197   endocytic membrane out of the enlarged compartments (Kuronita et al., 2002).

198   SCARB2 deficiency caused ureteric pelvic junction obstruction, deafness, and

199   peripheral neuropathuy in mice (Gamp et al., 2003). SCARB2 is expressed ubiquitously

200   in human tissues (Eskelinen et al., 2003); therefore, it might be involved in systemic

201   EV71 infections (Yamayoshi et al., 2009).

202

203   Human SCARB2 has 10 potential *N*-glycosylation sites (Fujita et al., 1992). But the

204   carbohydrate chains of human SCARB2 are not essential for the interaction between

205   EV71 and human SCARB2 (Yamayoshi and Koike, 2011). Experiments using a series

206   of chimeric proteins between human and mouse SCARB2 identified that the amino

207   acids 142 to 204 of human SCARB2 (encoded by human *SCARB2* exon 4) are

208   responsible for EV71 binding and infection (Yamayoshi and Koike, 2011).

209

210   L929 cells expressing human SCARB2 in the presence of puromycin (L-SCARB2 cells)

211   permitted the replication of all EV71 strains tested, including the non-PB strains

212   (Yamayoshi et al., 2009). CVA16 induced CPE in L-SCARB2 cells, whereas CVA2,

213   CVA3, CVA4, CVA5, CVA6, CVA8 and CVA12 did not. CVA16 grew efficiently in

214   L-SCARB2, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8 and CVA12 did not

215   (Table 1). Yamayoshi et al. (2009) concluded that CVA16 also infect L-SCARB2 cells

216   in a SCARB2-dependent manner and that infection with most other HEV-A is not

217   dependent upon SCARB2. CVA7, CVA10 and CVA14 induced CPE in both L-Empty

218   cells and L-SCARB2 cells (Yamayoshi et al., 2009). They could not to determine

219   whether the CPE induced by these viruses were due to hSCARB2–mediated infection.

220

221   **4. Annexin II**

222   Yang et al. (2011) identified annexin II as an EV71 VP1-binding protein on RD cells.

223   Using a recombinant VP1 protein of EV71 fused with a calmodulin-binding peptide

224   (VP1-CBP), they tried to identify VP1-binding proteins from the total cellular proteins

225   of RD cells. A virus-overlay protein-binding assay followed by a mass spectrometory

226   analysis identified annexin II as a VP1-binding protein.

227

228   Annexin II is a member of the annexin family – the multifunctional

229   phospholipid-binding proteins. Annexin II on the surface of endothelial cells acts as a

230   profibrinolytic coreceptor for both plasminogen and tissue plasminogen activator

231   facilitating the generation of plasmin (Kim and Hajjar, 2002). The interaction to annexin

232   II was specific to EV71; CVA16 did not bind to annexin II in the virus-overlay

233   protein-binding assay (Yang et al., 2011).

234

235   **5. Sialic acid**

236   Sialic acid (SA) is usually found as terminal monosaccharides on the glycan chains of

237   glycolipids and glycoproteins (Varki and Varki, 2007). Coxsackievirus A24 variant

238   (CVA24v) uses SA-containing glycoconjugates as attachment receptors on corneal cells

239   (Nilsson et al., 2008). Yang et al. (2009) hypothesized that SA would be important for

240   EV71 infection, as the transmission route of EV71 and CVA24v is fecal-oral and/or

241   droplet-aerosol route. EV71 infection to DLD-1 intestinal cells was inhibited by an

242   *O*-glycan synthesis inhibitor, but not by an *N*-glycan synthesis inhibitor. Sialidase

243   treatment decreased EV71 replication in DLD-1 cells. Furthermore, DLD-1 cells

244   co-cultured with SA-linked galactose significantly reduced the EV71 infection. Thus

245   Yang et al. (2009) concluded that SA-linked glycans are EV71 receptors on DLD-1 cells.

246   Recently, Neu5Acα2,3Gal disaccharides on PSGL-1 was reported as a candidate

247   receptor of CVA24v (Mistry et al., 2011). It is unknown whether other enteroviruses,

248   including HEV-A, recognize SA-containing glycans as the entry receptors.

249

250   **6. DC-SIGN**

251   DCs play crucial roles in antiviral immunity by functioning as professional

252   antigen-presenting cells to prime T cells and by secreting cytokines to modulate

253   immune responses. In a mouse model of EV71 infection, DCs from the brains of

254   EV71-infected, but not of uninfected, mice expressed viral antigen and primed T cells

255   efficiently (Lin et al., 2009a). Lin et al. (2009b) reported that EV71 infection enhances

256   mouse DCs to elicit protective immune response and also found that EV71 infects

257   human immature DCs and that viral entry is partially inhibited by anti-DC-SIGN

258   antibody. However, the direct interaction between EV71 and DC-SIGN is still unclear.

259   It is essential to characterize the role of DC-SIGN and other receptors for EV71 in DCs

260   for understanding the host immunological responses and immunopathogenesis of

261   HEV-A including EV71.

262

263   **7. Conclusion**

264   Identification of PSGL-1 and SCARB2 as the cellular receptors for EV71 and CVA16

265   has advanced our understanding of the early stages of HEV-A infections at the

266   molecular level. However, further experiments using clinical HEV-A isolates are

267   necessary to clarify the general role of PSGL-1 and SCARB2 in HEV-A infection and

268   their pathogenesis. Most of the prototype (laboratory-adapted) HEV-A strains other than

269   EV71 and CVA16 may use unidentified receptor(s) to infect susceptible human cells

270   such as RD cells. Characterization of the identified and unidentified HEV-A receptors is

271   essential to understand the mechanism of HEV-A infection and development of a

272   diverse array of the clinical outcomes of HEV-A-associated diseases.

273

274   **8. Acknowledgements**

280

281 **References**

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403

404 **Table 1**. Induction of CPE by the HEV-A strains.

Serotype	Strain	Accession No.	L-bsd[1]	L-PSGL-1.1	L-Empty[2,3]	L-SCARB2[3]
CVA2	Fleetwood	AY421760	-	-	-	-
CVA3	Olson	AY421761	-	-	-	-
CVA4	JR[3]	AB457644	-	-	-	-
CVA5	Swartz	AY421763	-	-	-	-
CVA6	Gdula	AY421764	-	-	-	-
CVA7	Parker	AY421765	-	+	+	+
CVA8	Donovan	AY421766	-	-	-	-
CVA10	Kowalik	AY421767	+	+	+	+
CVA12	Texas-12	AY421768	-	-	-	-
CVA14	G-14	AY421769	-	+	+	+
CVA16	G-10	U05876	-	+	-	+

405

406

407 1) Blasticidin-resistant L929 cells (a negative control for L-PSGL-1.1 cells)

408 2) Puromycin-resistant L929 cells (a negative control for L-SCARB2 cells)

409 3) Yamayoshi et al., 2009.

410 4) Prototype CVA4 strain (High Point) is unavailable from ATCC, therefore we used an

411 in-house reference strain of CVA4, the JR strain.

412

413 **Figure legend**

414

415 **Figure 1. HEV replication in L-PSGL-1.1 cells**

416 (A) Replication of the HEV-A strains (Table 1) in L-PSGL-1.1 cells in the presence or

417 absence of anti-PSGL-1 mAb (KPL1) or an isotype control. Cells were inoculated with

418 viruses at 10 $CCID_{50}$/cell for 1 h, washed, and incubated in the medium, as described

419 previously (Nishimura et al., 2009). Cell were incubated at 34°C. For mAb inhibition,

420 the cells were pretreated with 10 μg/ml mAb for 1 h, washed, and maintained in the

421 medium with 10 μg/ml mAb. At the indicated time (just after infection (0 h) and six

422 days postinfection (6 d)), the infected cells and supernatants were freeze-thawed and

423 viral titers were determined by $CCID_{50}$ titration using RD cells. The titers are expressed

424 as the mean and error bars indicate SD of triplicate analyses. The mean viral titers were

425 compared using Student's *t*-test. *P* values < 0.01 were considered statistically

426 significant.

427 (B) Viral replication of HEV-B, C, and D in L-PSGL-1.1 cells. Replication of two

428 HEV-B (CVB3-Nancy and echovirus 7(E7)-Wallace) and two HEV-C strains

429 (CVA21-Coe and poliovirus 1 (PV1)-Sabin 1), and one HEV-D (EV70-J670/71) strains

430 in L-PSGL-1.1 cells in the presence or absence of KPL1 or an isotype control. The titers

431 are expressed as the mean and error bars indicate SD of triplicate analyses. The mean

432 viral titers were compared using Student's *t*-test. *P* values < 0.01 were considered

433 statistically significant.

Figure 1

(A) CVA2, CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14; (B) CVB3, E7, CVA21, PV1, EV70. Viral titer ($\log_{10}$ CCID$_{50}$ per 100 μl) measured at 0 h and 6 d.

Legend: $P \geq 0.01$; $P < 0.01$
L-bsd; L-PSGL-1.1; L-PSGL-1.1 + isotype control; L-PSGL-1.1 + anti-PSGL-1
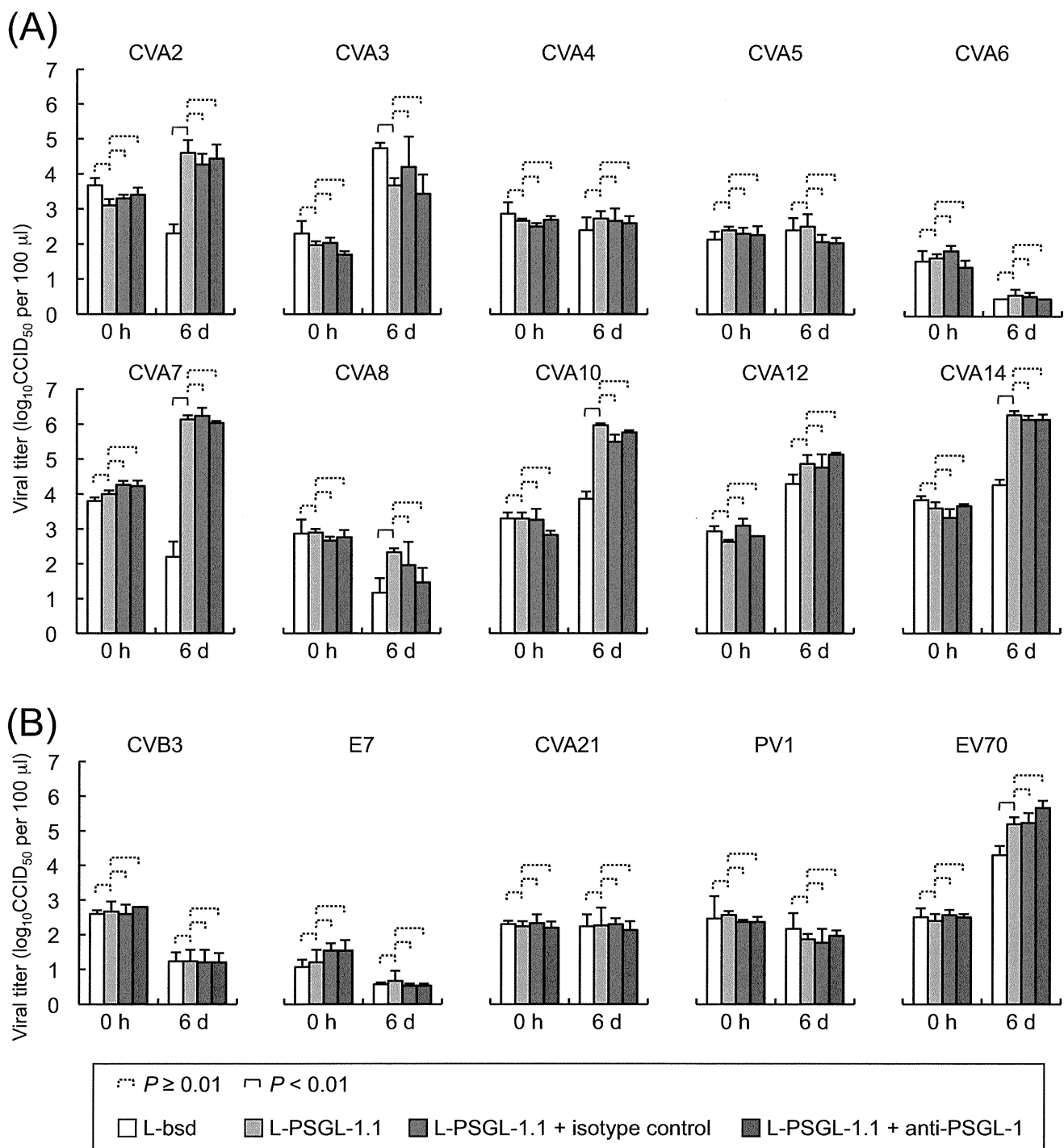
Tyrosine Sulfation of the Amino Terminus of PSGL-1 Is Critical for Enterovirus 71 Infection

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Abstract

Enterovirus 71 (EV71) is one of the major causative agents of hand, foot, and mouth disease, a common febrile disease in children; however, EV71 has been also associated with various neurological diseases including fatal cases in large EV71 outbreaks particularly in the Asia Pacific region. Recently we identified human P-selectin glycoprotein ligand-1 (PSGL-1) as a cellular receptor for entry and replication of EV71 in leukocytes. PSGL-1 is a sialomucin expressed on the surface of leukocytes, serves as a high affinity counterreceptor for selectins, and mediates leukocyte rolling on the endothelium. The PSGL-1–P-selectin interaction requires sulfation of at least one of three clustered tyrosines and an adjacent O-glycan expressing sialyl Lewis x in an N-terminal region of PSGL-1. To elucidate the molecular basis of the PSGL-1–EV71 interaction, we generated a series of PSGL-1 mutants and identified the post-translational modifications that are critical for binding of PSGL-1 to EV71. We expressed the PSGL-1 mutants in 293T cells and the transfected cells were assayed for their abilities to bind to EV71 by flow cytometry. We found that O-glycosylation on T57, which is critical for PSGL-1–selectin interaction, is not necessary for PSGL-1 binding to EV71. On the other hand, site-directed mutagenesis at one or more potential tyrosine sulfation sites in the N-terminal region of PSGL-1 significantly impaired PSGL-1 binding to EV71. Furthermore, an inhibitor of sulfation, sodium chlorate, blocked the PSGL-1–EV71 interaction and inhibited PSGL-1-mediated viral replication of EV71 in Jurkat T cells in a dose-dependent manner. Thus, the results presented in this study reveal that tyrosine sulfation, but not O-glycosylation, in the N-terminal region of PSGL-1 may facilitate virus entry and replication of EV71 in leukocytes.

Introduction

Enterovirus 71 (EV71) is a small, nonenveloped, positive-stranded RNA virus that belongs to human enterovirus species A of the genus *Enterovirus* in the family *Picornaviridae*. EV71 is a major causative agent of hand, foot, and mouth disease (HFMD), a common febrile disease affecting mainly young children. HFMD is characterized by a skin rash on the palms and soles, and ulcers on the oral mucosa. HFMD due to EV71 and other enteroviruses is usually mild and self-limited; however, EV71 infection may also cause severe neurological diseases including polio-like paralysis and fatal brainstem encephalitis in young children and infants (reviewed in [1,2]). Over the last decade, many EV71 outbreaks involving a number of fatal encephalitis cases have been reported throughout the world, especially in the Asia-Pacific region, including in Malaysia, Taiwan, Vietnam, and mainland China [2,3,4].

Using an expression cloning method by panning with a cDNA library from human Jurkat T cells, we recently identified human P-selectin glycoprotein ligand-1 (PSGL-1) as a functional cellular receptor for EV71 [5]. In addition, Yamayoshi et al. [6] identified scavenger receptor class B, member 2 (SCARB2) as another cellular receptor for EV71 by screening EV71-susceptible transformants after transfecting mouse L929 cells with genomic

DNA from human RD rhabdomyosarcoma cells. SCARB2 is ubiquitously expressed on a variety of tissues and cells [7], whereas the tissue distribution of PSGL-1 is mainly limited to immune cells such as leukocytes and dendritic cells [8]. We have also demonstrated that some EV71 strains (PSGL-1–binding strain; EV71-PB) use PSGL-1 as the primary and functional receptor for infection of Jurkat T cells, but other EV71 strains (PSGL-1–non-binding strain; EV71-non-PB) do not, suggesting phenotypic differences in PSGL-1 usage among EV71 strains. Thus, the identification of two distinct cellular receptors for EV71, PSGL-1 and SCARB2, has provided important clues in the elucidation of the molecular basis of early virus-host interactions and pathogenesis of EV71. However, little is known about the biological significance of the two EV71 receptors.

PSGL-1 is a sialomucin membrane protein that is expressed as a homodimer comprised of two disulfide-linked subunits. Interaction of PSGL-1 with selectins and chemokines is a key event during early inflammation of immune cells [8,9,10,11]. The N-terminal region of PSGL-1 is critical for PSGL-1 binding to P-, E- and L-selectins, and post-translational modifications such as O-glycosylation and tyrosine sulfation in the N-terminal region of PSGL-1 contribute the efficient binding to selectins [12,13,14,15]. We have previously shown that the N-terminal region of human PSGL-1 (amino acids 42–61) containing a potential O-glycosylation residue (T57) and three

Author Summary

Enterovirus 71 (EV71) is a major causative agent of hand, foot, and mouth disease and a diverse array of neurological diseases, including fatal encephalitis, in children. EV71 has increasingly caused large outbreaks of hand, foot, and mouth disease particularly in the Asia-Pacific region. Recently, we identified human P-selectin glycoprotein ligand-1 (PSGL-1) as a functional receptor for EV71. PSGL-1 on immune cells is a key molecule involved in early inflammatory events and the PSGL-1–selectin interaction is regulated by post-translational modifications of PSGL-1. Here, we found that a post-translational modification, tyrosine sulfation, at the N-terminal region of PSGL-1 is critical for its binding to EV71 and subsequent viral replication in lymphocytes. Important roles for tyrosine sulfation in protein-protein interactions have been widely accepted; however, involvement of tyrosine sulfation of the receptor in the virus-receptor interaction has been reported only for HIV-1. Therefore, this is the second and unique example of the involvement of tyrosine sulfation in specific virus-receptor interactions. Our results shed new light on biological roles for tyrosine-sulfated proteins in cell tropism and the pathogenesis of EV71.

potential tyrosine sulfation sites (Y46, Y48, and Y51) is directly responsible for PSGL-1 binding to EV71-PB [5]. Therefore, in the present study, we investigated the involvement of post-translational modifications of PSGL-1 in the binding to EV71-PB using a series of PSGL-1 mutants and an inhibitor of sulfation.

Tyrosine sulfation is an important late post-translational modification of secreted and membrane-bound proteins expressed in various mammalian cells and tissues and occurs in the trans-Golgi network [16,17]. Tyrosine sulfated proteins have been described in many mammalian species, and important roles for tyrosine sulfation in protein-protein interactions have been widely accepted, particularly for various chemokine receptors and their ligands that mediate leukocyte migration during inflammation. Furthermore, it has been well established that tyrosine sulfation of the N-terminal region of the chemokine receptor, C-C chemokine receptor 5 (CCR5), plays critical roles in the function of CCR5 as a coreceptor for virus entry and replication of CCR5-tropic human immunodeficiency virus type 1 (HIV-1) variants [18].

Here we demonstrate that tyrosine sulfation of the N-terminal region of PSGL-1 facilitates PSGL-1–EV71 interaction and viral replication of EV71-PB in Jurkat T cells. To our knowledge, this is the second direct example of the involvement of tyrosine sulfation in specific virus-receptor interactions, a modification that mediates viral entry and replication in target cells.

Results

O-glycosylation at T57 of PSGL-1 is not necessary for EV71-1095 binding

For binding to selectins, PSGL-1 requires post-translational modifications with sialyl Lewis x-containing O-glycans at T57. α1,3-fucosyltransferase (FUT7) is involved in the biosynthesis of sialyl Lewis x determinants (Fig. 1A) [19,20]. Prevention of O-glycosylation by alanine substitution at T57 (T57A) eliminates binding of PSGL-1 to P-selectin without affecting tyrosine sulfation [12]. First, we generated and expressed a PSGL-1-T57A mutant (Fig. 1A) in 293T cells (293T/T57A) to examine the role of O-glycosylation on T57 for PSGL-1 binding to EV71-1095, a representative strain of EV71-PB [5]. As a positive binding control, we used a soluble form

of recombinant P-selectin (P-selectin-Fc). P-selectin-Fc did not bind to any PSGL-1 transfectants in the presence of 2 mM EDTA (Fig. 1B). P-selectin-Fc bound weakly to 293T cells transiently expressing PSGL-1 (293T/PSGL-1) in the presence of Ca^{2+} but not to 293T/T57A cells (Fig. 1B). Double expression of PSGL-1 and FUT7 in 293T cells resulted in the efficient binding of P-selectin-Fc to PSGL-1 in a calcium-dependent manner (Fig. 1B). Even in the presence of Ca^{2+} and FUT7, P-selectin-Fc did not bind to 293T/T57A cells (Fig. 1B). These observations are consistent with previous findings that interaction of PSGL-1 with P-selectin is calcium-dependent and requires appropriate O-glycosylation of PSGL-1 at T57 [10,12]. In contrast, EV71-1095 showed marked binding to 293T/PSGL-1 cells in a calcium-independent manner, even in the absence of FUT7 (Fig. 1B). EV71-1095 also bound to 293T/T57A cells (Fig. 1B). These results indicate that, unlike the interaction between PSGL-1 and P-selectin, the interaction between PSGL-1 and EV71-1095 does not require Ca^{2+} and the O-glycans at T57 of PSGL-1.

Sialic acids are not necessary for EV71-1095 binding

To examine the role of sialic acids on the cell surface, including sialyl Lewis x moieties in the potential O-glycans at T44 and T57 of PSGL-1, on EV71 binding to 293T/PSGL-1 cells, we tested EV71 binding to the cells pretreated with sialidase. Sialidase treatment removed cell-surface sialyl Lewis x (Fig. 2A) and reduced P-selectin-Fc binding to 293T/PSGL-1 cells (Fig. 2B). On the other hand, EV71-1095 binding to the sialidase-treated cells was not reduced regardless of the removal of sialyl Lewis x (Fig. 2C). Although treatment with sialidase decreased EV71 infection to DLD-1 cells [21], sialic acids on the cell surface of 293T/PSGL-1 cells are not necessary for the binding of PSGL-1 to EV71-1095.

An inhibitor of sulfation reduces PSGL-1 binding to EV71-PB

In addition to O-glycosylation of PSGL-1, sulfation of the three tyrosines (Y46, Y48, and Y51) in the N-terminal region of PSGL-1 is required for high affinity binding to P- and L-selectins [13,14,15,22,23]. To assess the role of tyrosine sulfation of PSGL-1 in the PSGL-1–EV71 interaction, we treated 293T/PSGL-1 cells with sodium chlorate, an inhibitor of sulfation that blocks PSGL-1 binding to P-selectin [13]. As described previously, sodium chlorate had no apparent effect on PSGL-1 expression on the cell surface (Fig. 3A). On the other hand, sodium chlorate reduced sulfated tyrosines on the cell surface (Fig. 3B) and inhibited EV71-1095 binding to 293T/PSGL-1 cells in a dose-dependent manner (Fig. 3C). These observations indicated that sulfation of PSGL-1, in addition to its expression on the cell surface, is important for EV71 binding.

One or more tyrosines in the N-terminal region of PSGL-1 are important for EV71-PB binding

We then determined the requirement for the putative sulfated tyrosines (Y46, Y48, or Y51) in the N-terminal region of PSGL-1 for its binding to EV71-1095. We generated PSGL-1 mutants with phenylalanine substitutions at one or more tyrosines and a mutant with a deletion of this region (Fig. 4A). We transfected 293T cells with expression plasmids containing the PSGL-1 mutants and used them for the EV71 binding assay using flow cytometry. 293T cells transfected with an empty vector expressed little or no detectable tyrosine sulfated proteins on the cell surface (Fig. 4B). Similar to the binding of PSGL-1 to P-selectin [13,14], substitution of the tyrosines with phenylalanine prevented tyrosine sulfation and
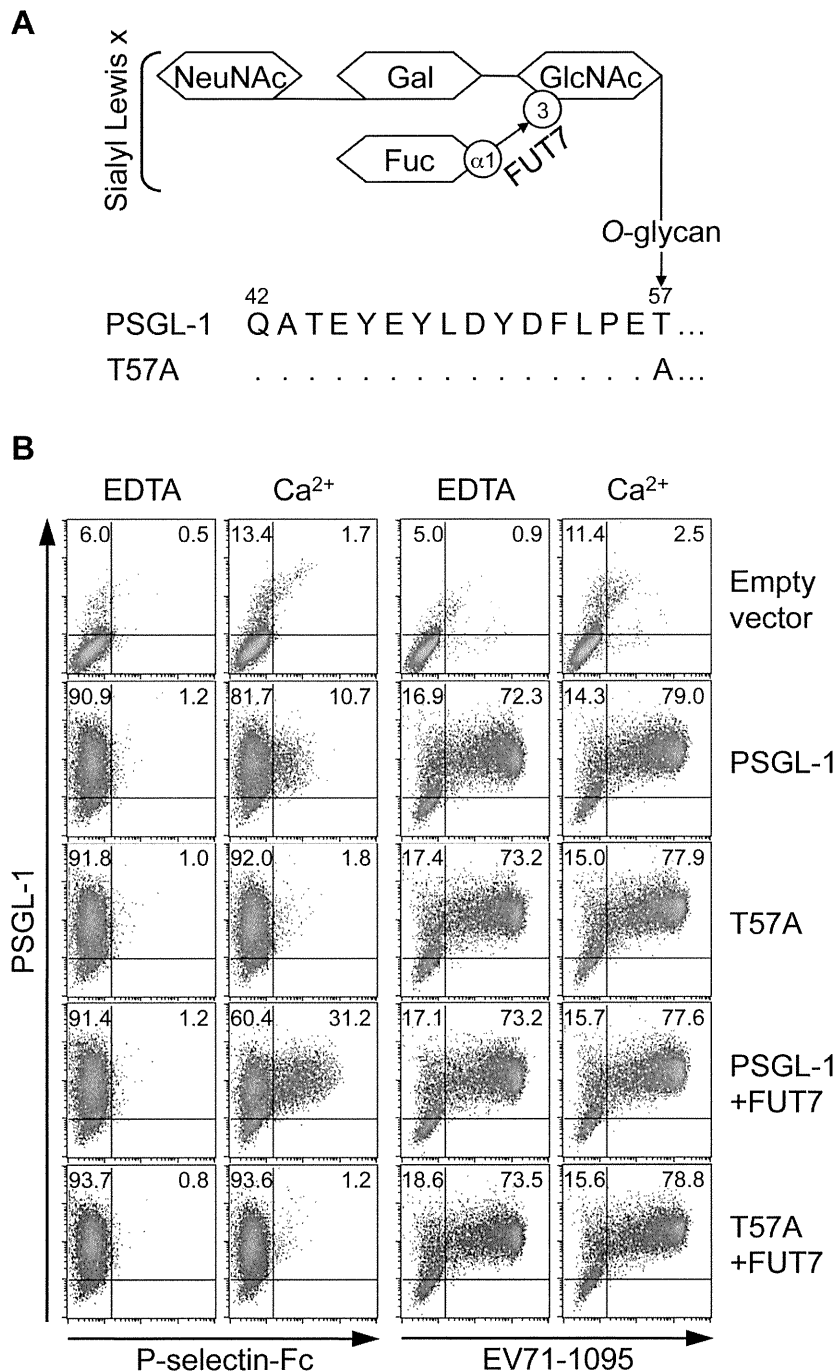
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Figure 1. PSGL-1 O-glycosylation at T57 is not necessary for binding to EV71-1095. (A) Schematic structure of the O-glycosylation of PSGL-1 and the T57A mutant. FUT7 is involved in the synthesis of sialyl Lewis x. (B) 293T cells were transfected with the indicated expression plasmids. Transfectants were incubated with P-selectin-Fc or EV71-1095 in the presence (Ca^{2+}) or absence (EDTA) of 2 mM $CaCl_2$ followed by the P-selectin-Fc or EV71 binding assay using flow cytometry. The percentage of cells bound to P-selectin-Fc or EV71-1095 is indicated in the upper right quadrant. The data are representative of three independent experiments.
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PSGL-1 binding to EV71-1095 (Fig. 4B). Substitution of one or two tyrosines slightly reduced (Y46F) or impaired (Y48F, Y51F, Y4648F, or Y4651F) the binding of PSGL-1 to EV71-1095 regardless of the apparent expression of tyrosine sulfated proteins on the cell surface (Fig. 4B). Substitution of two or three tyrosines (Y4851F or FFF) or deletion of the region (d46–51) reduced tyrosine sulfated proteins on the cell surface and completely disrupted the PSGL-1–EV71 interaction (Fig. 4B). We also examined the role of tyrosine sulfation in PSGL-1 binding to other EV71-PB strains. Binding of SK-EV006, C7/Osaka, KED005, and 75-Yamagata strains to 293T/PSGL-1 cells was also inhibited by sodium chlorate (Fig. S1). These strains bound to 293T/T57A cells but not to 293T cells expressing the PSGL-1-FFF mutant. Taken together, these findings demonstrate that, in contrast to O-glycosylation at T57, tyrosine sulfation of PSGL-1 is essential for the efficient binding to EV71-PB strains.
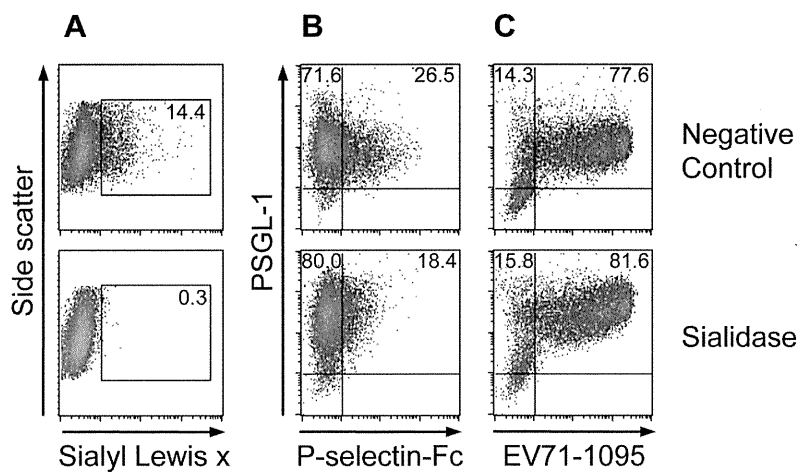
Figure 2. Effect of sialidase treatment on PSGL-1 binding to EV71-1095. (A) Sialyl Lewis x expression on the cell surface, as measured with flow cytometry. The percentage of cells expressing sialyl Lewis x is indicated. (B) The cells were examined with the P-selectin-Fc binding assay using flow cytometry. The percentage of cells bound to P-selectin-Fc is indicated in the upper right quadrant. (C) The cells were examined with the EV71 binding assay using flow cytometry. The percentage of cells bound to EV71-1095 is indicated in the upper right quadrant. As a negative control, 293T/PSGL-1 cells were incubated in the medium without sialidase. The data are representative of three independent experiments.
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Sodium chlorate inhibits EV71-PB replication in Jurkat T cells

We next examined whether sulfation of PSGL-1 is required for PSGL-1-dependent replication of EV71-PB in Jurkat T cells. Jurkat T cells were infected with EV71 and cultured in the presence of sodium chlorate to inhibit the sulfation of PSGL-1. Sodium chlorate treatment did not affect PSGL-1 expression on Jurkat T cells (Fig. 5A). On the other hand, sodium chlorate significantly inhibited the replication of EV71-1095 in a dose-

dependent manner (Fig. 5B). The replication of other EV71-PB strains was also inhibited in the presence of sodium chlorate (Fig. 6). In contrast, replication of EV71-non-PB strains (EV71-02362 and EV71-Nagoya), which can replicate in Jurkat T cells in a PSGL-1-independent manner [5], was not affected by sodium chlorate (Figs. 5C and 6). This observation supports that sodium chlorate inhibited replication by blocking EV71-PB entry into the cells. To confirm that sodium chlorate is acting at the receptor level, we transfected Jurkat T cells with genomic RNA of EV71-
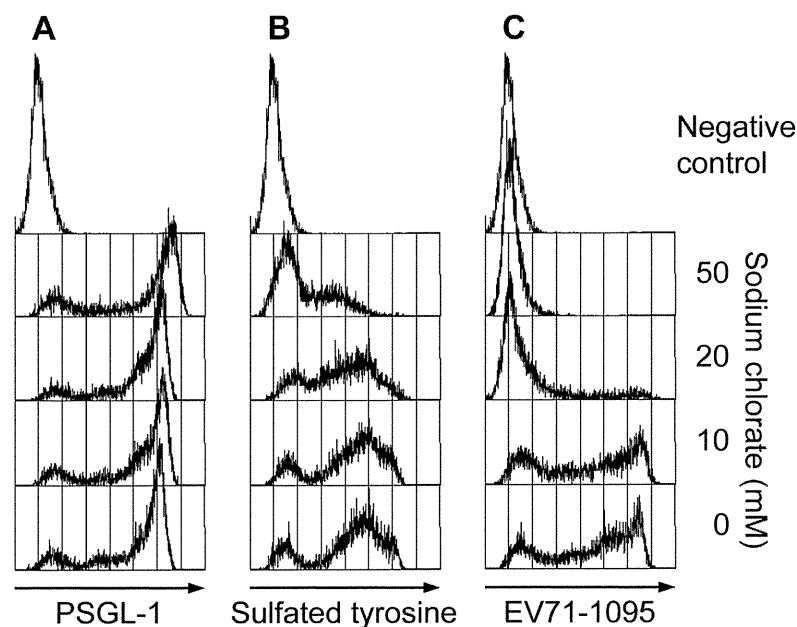

Figure 3. Effect of sodium chlorate on PSGL-1 binding to EV71-1095. Pretreatment of 293T/PSGL-1 cells with sodium chlorate reduces EV71-1095 binding in a dose-dependent manner. (A) PSGL-1 expression on the cell surface, as measured with flow cytometry. As a negative control, 293T/PSGL-1 cells cultured in the absence of sodium chlorate were stained with an isotype control antibody. (B) Sulfated tyrosines on the cell surface, as measured with flow cytometry. As a negative control, 293T/PSGL-1 cells cultured in the absence of sodium chlorate were stained with an isotype control antibody. (C) The cells were examined with the EV71 binding assay using flow cytometry. As a binding control, 293T/PSGL-1 cells were treated with mock-infected culture supernatant. The data are representative of three independent experiments.
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A

Tyrosine sulfation

		42	46	48	51	57
PSGL-1		Q A T E Y E Y L D Y D F L P E T ...

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              42     46  48   51            57
PSGL-1    Q A T E Y E Y L D Y D F L P E T ...
Y46F      . . . . F . . . . . . . . . . ...
Y48F      . . . . . . F . . . 1 . . . . . ...
Y51F      . . . . . . . . . F . . . . . ...
Y4648F    . . . . F . F . . . . . . . . ...
Y4651F    . . . . F . . . . F . . . . . ...
Y4851F    . . . . . . F . . F . . . . . ...
FFF       . . . . F . F . . F . . . . . ...
d46-51    . . . . – – – – – – . . . . . ...
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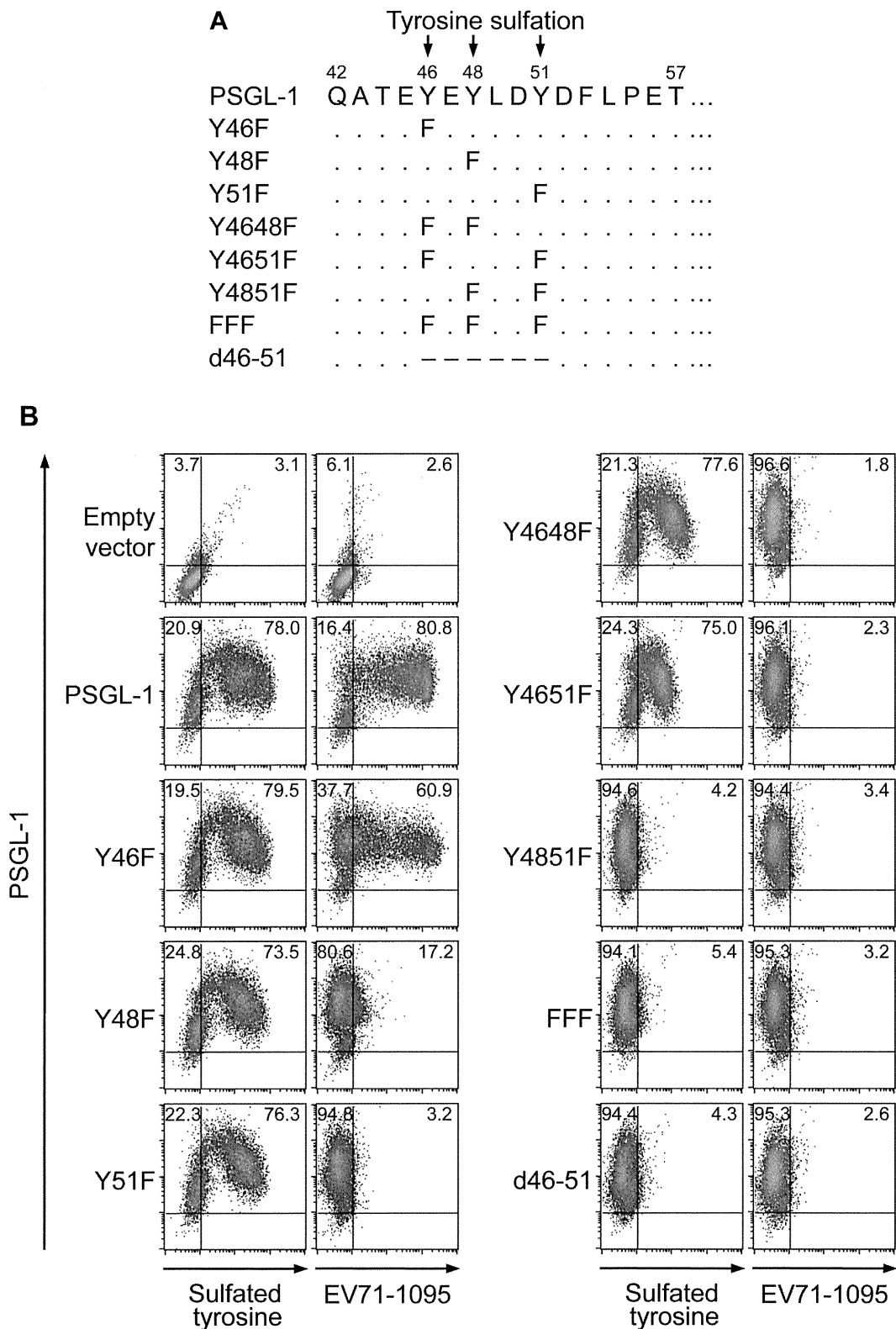
B

Figure 4. Sulfated tyrosines in the N-terminal region of PSGL-1 are important for binding to EV71-1095. (A) Putative sites of tyrosine sulfation (Y46, Y48, and Y51) in the N-terminus (aa 42–57) of PSGL-1 and the series of PSGL-1 mutants constructed. Identical and deleted amino acids are indicated by dots (.) and dashes (–), respectively. (B) EV71-1095 binding to PSGL-1 mutants. The percentage of cells expressing tyrosine sulfated proteins or bound to EV71-1095 is indicated in the upper right quadrant. The data are representative of three independent experiments.
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