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Heparin-binding domain of bovid herpesvirus 1 glycoprotein gIII

Brief Report

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Summary. Bovid herpesvirus 1 (BHV-1) glycoprotein gIII functions as a major virus attachment protein through binding to a heparinlike moiety on the host cells. To identify the functional domain, a panel of gIII deletion mutants was constructed, expressed in COS-7 cells, and examined for heparin-binding activity. Mutants with deletion of amino acid residues 103–173 and 324–443 bound to heparin as well as full-length gIII, whereas a mutant with residues 172–337 deleted showed no binding to heparin. In another mutant, with residues 172–211 deleted, the activity was reduced by one-third. These data suggest that the amino acid sequence between residues 172 and 323 contains the functional domain of BHV-1 gIII for heparin-binding and that especially the sequence between residues 212–323 includes a critical site for the activity.

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The initial event in the life cycle of a virus is attachment to a specific receptor on a host cell. Our previous studies showed that glycoprotein gIII of bovid herpesvirus 1 (BHV-1) functions as a major viral attachment protein [11], and that the attachment is mediated by the interaction between the glycoprotein and a heparinlike moiety on the plasma membrane independently of other virus components, such as glycoproteins gI and gIV [9, 14]. In contrast, it has been shown that both gC (a gIII homolog) and gB of herpes simplex virus separately bind to heparin [6] and that pseudorabies virus (PRV) gIII binds to heparin together with gII [10]. It is therefore thought that the mechanisms by which these viruses attach to a heparinlike moiety on the cells might differ, even though all gIII homologs play a predominant role in virus attachment.

BHV-1 gIII consists of 521 amino acid residues which compose a signal peptide between residues 7 and 21, a projecting region, and the transmembrane anchor domain between residues 467 and 500 followed by a hydrophilic

C-terminal [3]. The projecting region of gIII contains several clusters of basic amino acid residues which have been postulated to mediate the binding of the glycoprotein to heparin. In the present study, to determine the heparin-binding domain of BHV-1 gIII, a panel of deletion mutants was expressed in transfected COS-7 cells and their heparin-binding activity was examined.

The 2.0-kbp *Bam*HI/*Sma*I fragment containing the entire region of BHV-1 gIII was excised from pVI85, of which subclone pSRBH3-456 was previously used for expression of the gIII [12], and cloned into the *Bam*HI/*Eco*RV sites in the multicloning site of the pcDNAI/Neo vector (Invitrogen Co., San Diego, CA, U.S.A.), to create full-length gIII-expressing plasmid pcDNAgIII. Then the gIII gene was excised from pcDNAgIII as a *Hin*dIII/*Xba*I fragment and subcloned into pTFl [17]. The resultant plasmid, pTFgIII, was digested with *Ava*I and *Sac*I and treated with T4 DNA polymerase, and the repaired ends were ligated to construct the mutant plasmid pTFd103–173. Plasmid pTFd172–211 was generated by digestion of pTFgIII with *Sac*I and partially with *Pvu*I, blunt-end-repairing, and insertion of 10-bp *Bgl*II linker GAAGATCTTC. To construct pTFd171–337, pTFgIII was digested with *Sac*I and *Xho*I and blunt-end-repaired, and the ends were ligated. Plasmid pTFd324–443 was generated by digestion of pTFgIII with *Sal*I and by self-ligation. The mutant plasmids pTFd103–173, pTFd172–211, pTFd171–337, and pTFd324–443 were digested with *Hin*dIII and *Xba*I, and the inserts were cloned into the pcDNAI/Neo vector to generate a panel of expression vectors for deleted gIII. Plasmid pcDNAd103–173 encoded a protein designated gIIId103–173, which lacked residues 103 to 173 of gIII. For plasmid pcDNAd172–211, 40 amino acids of gIII between residues 172 and 211 were removed and an amino acid sequence Gly-Arg-Ser-Ser was inserted at the junction created by the *Bgl*II linker insertion. The expressed protein was designated gIIId172–211. Plasmid pcDNAd172–337 encoded gIIId172–337, which lacked residues 172 to 337 and received an insertion of Val after residue 172. Plasmid pcDNAd324–443 encoded a protein lacking residues 324 to 443 (gIIId324–443). No residue was inserted into gIIId103–173 or gIIId324–443. Positions of deletion and insertion of the mutants described above are depicted in Fig. 1.

COS-7 cells in 6-well microplates were transfected with 5–10 μg of plasmid DNA by using Lipofectin Reagent (Bethesda Research Laboratories Inc., Gaithersburg, MD, U.S.A.), according to the manufacturer's instructions. Sixty hours later, the cells were fixed with methanol-acetone (1:1), fluorescent-antibody (FA) – stained with rabbit hyperimmune serum against BHV-1, and examined for expression of gIII as previously described [13]. As shown in Fig. 1, specific fluorescence was observed in the cells transfected with all of the plasmids tested. The results indicate that deleted gIIIs as well as full-length gIII were expressed in the transfected cells and that the deleted gIIIs still retained antigenic authenticity. As previously found by using the SRα promoter system [12], not only full-length gIII but also deleted gIIIs were present in the cytoplasm of the cells. No accumulation of the gIIIs was observed in the nucleus, although Fitzpatrick et al. [4] reported nuclear accumulation of recombinant gIII in murine cells in their stable expression system.

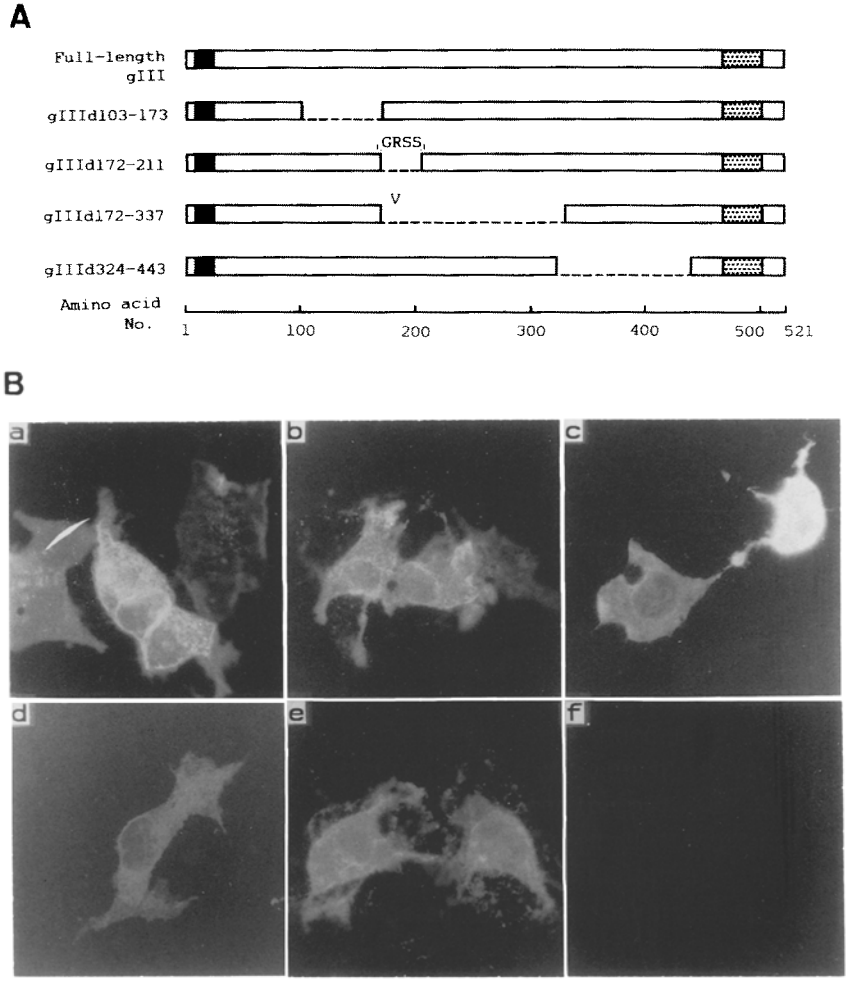

Fig. 1. Characterization of gIII deletion mutants. **A** Wild-type gIII protein of BHV-1 and deletion mutants. Solid and dotted boxes represent the signal sequence and the transmembrane anchor of gIII, respectively. The relative sizes and positions of the deletions within gIII are shown by the broken lines. Mutants gIIId172–211 and gIIId172–337 contain insertional amino acid sequences Gly-Arg-Ser-Ser (*GRSS*) and Val (*V*), respectively, at the junctions. **B** Expression of the wild-type gIII and the gIII deletion mutants. COS-7 cells transfected with pcDNAgIII (*a*), pcDNAd103–173 (*b*), pcDNAd172–211 (*c*), pcDNAd172–337 (*d*), pcDNAd324–443 (*e*), or pcDNAI/Neo (*f*) were incubated with anti-BHV-1 rabbit hyperimmune serum then stained with fluorescein-isothiocyanate-labeled anti-rabbit IgG

To quantitate the expressed gIIIs, 5-μl portions of serial 2-fold dilutions of cell lysate were dotted on a PVDF membrane (Nihon Millipore Ltd., Tokyo, Japan) and immunoenzymatically stained with the rabbit immune serum. The results showed that all of the gIIIs extracted from the cells were recognized by the antibodies (Fig. 2). A mixture of each cell lysate and 1 000 units of heparin was also quantified to ascertain if the immune serum effectively recognized the gIII in the presence of heparin. No lysate showed reduced reactivity (data not shown), indicating that the predicted gIII-heparin complexes could be recognized

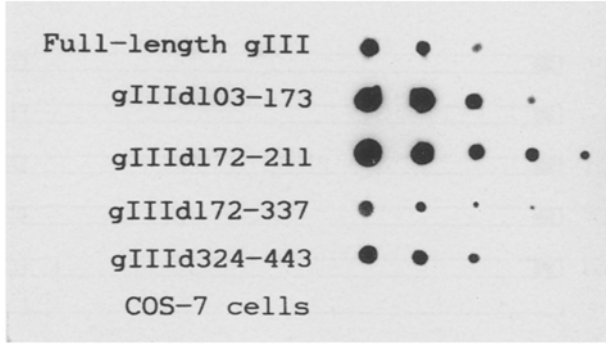

Fig. 2. Quantitation of gIII deletion mutants in COS-7 cells. Serial 2-fold dilutions of lysates made from the transfected cells by extraction with 1% Triton X-100 were dotted on a PVDF membrane and incubated with anti-BHV-1 rabbit hyperimmune serum and with alkaline phosphatase-labeled anti-rabbit IgG. The antigens were visualized by the addition of bromochlorindolyl phosphate and nitroblue tetrazolium chloride as substrates

by the immune serum. The titers of the gIIIs were then expressed as the reciprocals of the maximal dilution detected in this assay. Full-length gIII, gIIId103–173, and gIIId324–443 were usually produced at a level between 1 800 and 2 400 units per well, and gIIId172–211 and gIIId172–337 at a level between 800 and 1 200 units per well.

The heparin-binding activity of the gIIIs was assessed by incubation of the lysate containing approximately 500 units of the gIIIs with heparin-agarose beads (Pierce Chemical Co., Rockford, IL, U.S.A.), followed by detection of the gIIIs on the beads with the rabbit immune serum. Figure 3 shows that gIIId172–337 had no heparin-binding activity. Mutant gIIId172–211, whose deletion overlapped that of gIIId172–337, was also shown to have reduced activity. No reduction was observed for gIIId103–173 or gIIId324–443. An inhibition assay was also carried out by prior incubation of the lysate with heparin. Marked reduction was observed for gIII, gIIId103–173, gIIId172–211, and gIIId324–443. These findings demonstrated that a panel of deletion mutants including gIIId103–173, gIIId172–211, and gIIId324–443 as well as full-length gIII specifically bound to the heparin-beads.

The results presented here suggest that at least amino acid residues 172 to 337 of gIII are involved in the heparin-binding activity of the glycoprotein. Since gIIId324–443 showed no reduction of the activity, amino acid residues 324 to 337 of gIII may not be involved in the binding. gIIId172–211 was also able to bind to heparin, although the activity was reduced. Therefore, it is postulated that an amino acid sequence between residues 212 and 323 contains a functionally critical domain for the binding of BHV-1 gIII to heparin molecules. It is worth noting that this region, especially residues 263 to 337 is highly conserved among gIII homologs of other alphaherpesviruses [1, 7, 15, 16]. Amino acid residues 172 to 211 seemed to take part in the binding but not to be essential for the activity.

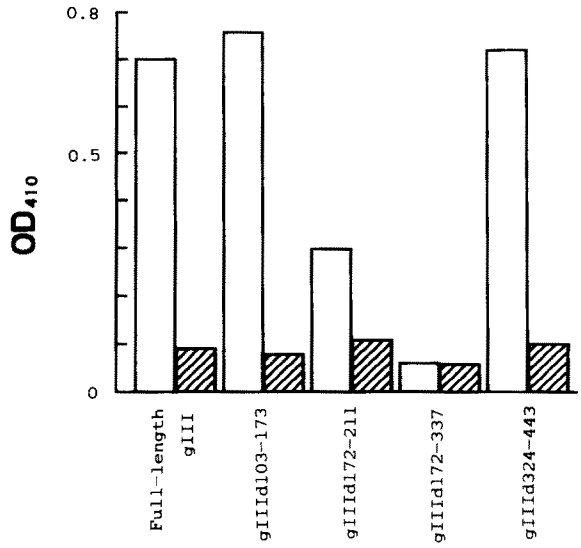

Fig. 3. Heparin-binding activity of gIII deletion mutants. Each cell lysate containing approximately 500 units of gIII was mixed with heparin-agarose beads and incubated with anti-BHV-1 rabbit hyperimmune serum and with alkaline phosphatase-labeled anti-rabbit IgG, then nitro-phenyl phosphate was added as a substrate. The quantity of gIII binding to the beads in the absence of exogenous heparin (□) or in the presence of 1000 units of heparin (▨) was measured by absorbance at 410 nm

Recently Liang et al. [9] defined 5 heparin-binding sites between residues 129 and 310 of BHV-1 gIII by using synthetic peptides. Among them, however, only one peptide, containing residues 297 to 310, inhibited plaque formation and adsorption of virus to the cells. Of the other 8 peptides reported to bind to heparin, 3 amino acid sequences were included between residues 212 and 323, whereas 5 were not. The present findings seem to be consistent with the results of the inhibition assays rather than those of the binding assay reported by Liang et al. [9]. It was also noted that the accessary sequence between residues 172 and 211 was included within a region which was reported to determine the sensitivity of gIII to heparin [9].

We previously speculated that a cluster of basic amino acids including Lys and Arg between residues 114 and 144 of gIII was responsible for heparin binding because of an electrostatic interaction, presumably mediated by the positive charge of the amino acid sequence and the negative charge of the carbohydrate chains of heparin [14]. Although this region was defined as one of the heparin-binding sites by Liang et al. [9], gIIId103–173 lacking this region bound to heparin as effectively as full-length gIII. As shown in Fig. 4, BHV-1 gIII is predicted to contain 4 clusters (I to IV) of positively charged residues, such as Arg and Lys, in the projecting region. The present data suggest that clusters III and/or IV are critical for the heparin-binding activity of gIII. In contrast, cluster I did not seem to take part in the binding. Cluster II appeared to reinforce the interaction between heparin and the glycoprotein.

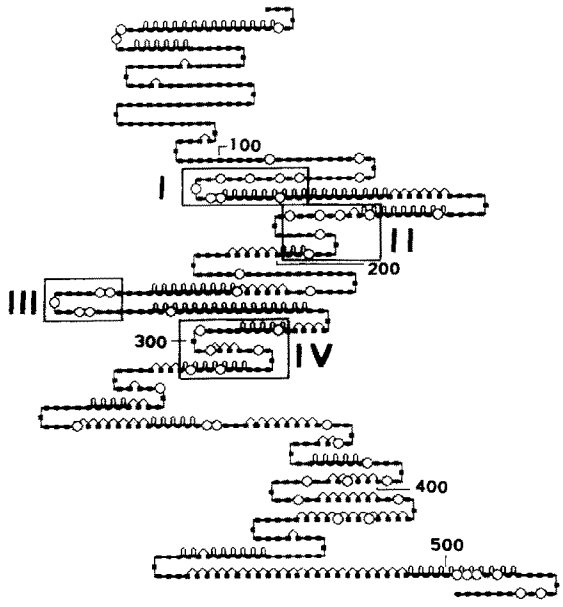

Fig. 4. Positively charged clusters on the secondary structure prediction of BHV-1 gIII. Chou-Fasman prediction [2] of gIII was carried out according to Fitzpatrick et al. [3]. The probabilities of the occurrence of α-helices ( ), β-sheets ( ), random coil ( ), and β-turn ( ) were determined with the DNASIS version 2 program (Hitachi Software Engineering Co., Tokyo, Japan). The open circles in the prediction represent Arg or Lys of the gIII sequence. The clusters of positively charged amino acids are enclosed by boxes

PRV gIII has also been shown to function as a major attachment protein by interacting with a heparinlike moiety on the cells [10]. A recent study using a panel of deletion mutants demonstrated that the amino-terminal third of PRV gIII is responsible for attachment of virions to cells as well as for sensitivity to heparin [5]. Although BHV-1 and PRV gIII are structurally homologous and functionally complementary [8], the functional domains of the glycoproteins did not coincide. This discrepancy might be a reflection of different sensitivity to heparin or possibly different mechanisms for attachment of the viruses to a heparinlike moiety on the cells.

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