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A Single-Amino-Acid Substitution in Herpes Simplex Virus 1 Envelope Glycoprotein B at a Site Required for Binding to the Paired Immunoglobulin-Like Type 2 Receptor α (PILRα) Abrogates PILRα-Dependent Viral Entry and Reduces Pathogenesis▽

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Paired immunoglobulin-like type 2 receptor α (PILRα) is a herpes simplex virus 1 (HSV-1) entry receptor that associates with O-glycans on HSV-1 envelope glycoprotein B (gB). Two threonine residues (Thr-53 and Thr-480) in gB, which are required for the addition of the principal gB O-glycans, are essential for binding to soluble PILRα. However, the role of the two threonines in PILRα-dependent viral entry remains to be elucidated. Therefore, we constructed a recombinant HSV-1 carrying an alanine replacement of gB Thr-53 alone (gB-T53A) or of both gB Thr-53 and Thr-480 (gB-T53/480A) and demonstrated that these mutations abrogated viral entry in CHO cells expressing PILRα. In contrast, the mutations had no effect on viral entry in CHO cells expressing known host cell receptors for HSV-1 gD, viral entry in HL60 cells expressing myelin-associated glycoprotein (MAG) (another HSV-1 gB receptor), viral attachment to heparan sulfate, and viral replication in PILRα-negative cells. These results support the hypothesis that gB Thr-53 and Thr-480 as well as gB O-glycosylation, probably at these sites, are critical for PILRα-dependent viral entry. Interestingly, following corneal inoculation in mice, the gB-T53A and gB-T53/480A mutations significantly reduced viral replication in the cornea, the development of herpes stroma keratitis, and neuroinvasiveness. The abilities of HSV-1 to enter cells in a PILRα-dependent manner and to acquire specific carbohydrates on gB are therefore linked to an increase in viral replication and virulence in the experimental murine model.

Herpes simplex virus 1 (HSV-1) entry into host cells depends on interactions between cell surface receptors and HSV-1 virion envelope glycoproteins (39). Five of the 12 HSV-1 envelope glycoproteins that have been identified thus far (i.e., glycoprotein B [gB], gC, gD, gH, and gL) have roles in viral entry (39). Both gB and gC mediate virion attachment by interacting with cell surface glycosaminoglycan, primarily heparan sulfate (16, 17). Although not essential for entry, this step provides stable interactions between the virion and the cell that favor the next steps (39). These steps include gD binding to one of its identified receptors, i.e., herpesvirus entry mediator (HVEM), nectin-1, and specific sites on heparan sulfate 3-*O*-sulfated heparan sulfate (3-*O*-S-HS) generated by certain 3-*O*-sulfotransferases (3-*O*-STs) (14, 28, 38, 51). Subsequent fusion between the virion envelope and host cell membrane, which requires the cooperative function of gB, heterodimer gH/gL, gD, and a gD receptor, then produces nucleocapsid penetration into the cell (31, 46).

In addition to the interaction of gD with a gD receptor, gB binding to a cellular receptor other than heparan sulfate has been suggested to mediate viral entry, based on the observation that a soluble form of gB binds to heparan sulfate-deficient cells and blocks HSV-1 infection of some cell lines (3). Consistent with this observation, we have reported that paired immunoglobulin-like type 2 receptor α (PILRα) associates with gB and functions as an HSV-1 entry receptor (36). Viral entry via PILRα appears to be conserved among alphaherpesviruses, but there is a PILRα preference based on the observation that PILRα is able to mediate the entry of pseudorabies virus, a porcine alphaherpesvirus, but not of HSV-2 (1). Importantly, HSV-1 infection of human primary monocytes expressing both HVEM and PILRα was blocked by either an

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anti-PILRα or anti-HVEM antibody, suggesting that cellular receptors for both gD and gB are required for HSV-1 infection (36). However, CHO-K1 cells, which are resistant to HSV-1 infection, can become susceptible to HSV-1 entry and HSV-1-induced cell fusion after the overexpression of either a gD receptor, such as nectin-1, or PILRα (14, 36). It was thought that CHO-K1 cells express endogenously low levels of gB and gD receptors that allow the single overexpression of either a gB or gD receptor to support detectable levels of HSV-1 entry and HSV-1-induced cell fusion (36). More recently, myelin-associated glycoprotein (MAG), which has homology to PILRα, was also reported to serve as the gB receptor for HSV-1 and varicella-zoster virus (40). However, the importance of PILRα- or MAG-dependent viral entry in HSV-1 infection and pathogenesis *in vivo* remains to be elucidated.

PILRα is one of the paired receptor families, in which one receptor has inhibitory functions and the other mediates activation functions, and is expressed mainly in immune system cells (13, 29). In addition, PILRα was previously reported to be expressed in certain types of cells in neural tissues (36). We previously identified one of the PILRα ligands as CD99 (37). Interestingly, PILRα recognition of CD99 is dependent on the addition of sialylated O-linked sugar chains at particular CD99 threonines (50). Similarly, we recently demonstrated that a specific sialylated O-glycan(s) on gB is critical for PILRα binding, based on observations that neuraminidase, which removes sialic acid, and benzyl-α-GalNAc treatment, which blocks O-glycan synthesis, inhibited gB binding to a soluble PILRα (49). More importantly, one (Thr-53) or both (Thr-53 and Thr-480) putative O-glycosylation sites identified by bioinformatics analysis are required for the binding of gB to soluble PILRα, and the replacement of both Thr-53 and Thr-480 with alanine significantly inhibited the addition of O-glycans to gB (49). These observations suggest that Thr-53 and Thr-480 in gB are O-glycosylated, and these sites, and probably the addition of specific carbohydrates to them, are required for the interaction of gB with PILRα. However, it remains uncertain whether gB Thr-53 and Thr-480, and probably the gB O-glycosylation of these sites, are required for PILRα-dependent viral entry in natural infections.

In the present study, we have shown that the alanine replacement of gB Thr-53 (gB-T53A) alone or of both gB Thr-53 and Thr-480 (gB-T53/480A) significantly inhibited cell-cell fusion in CHO cells expressing PILRα, gB, gD, gH, and gL, whereas the mutations had no effect on cell-cell fusion in CHO cells expressing nectin-1, gB, gD, gH, and gL. Furthermore, we constructed recombinant HSV-1 carrying the gB-T53A and gB-T53/480A mutations and found that these mutations abrogated PILRα-dependent viral entry but had no effect on viral entry via known receptors for HSV-1 gD and MAG, viral attachment to heparan sulfate, and viral replication in PILRα-negative cells. We also tested these recombinant viruses in mice and present data showing that the mutations in gB significantly reduced viral replication, the development of herpes stromal keratitis (HSK), and neuroinvasiveness.

MATERIALS AND METHODS

Cells and viruses. Vero, HEL, CHO-K1, IC21, CHO-hPILRα, CHO-mPILRα, CHO-hNectin-1, and CHO-neo cells were described previously (1, 19, 36, 42). Colon 26 (CT26) cells, which are murine colon epithelial tumor cells, were described previously (7, 23). CHO-m3OST and CHO-hHVEM cells were generated by the transfection of CHO-K1 cells with pMXs-IRES-human-CD8 retrovirus expression vectors containing mouse heparan sulfate 3-*O*-ST 3B1 and human HVEM cDNAs, respectively, and the sorting of the CD8-positive cells by fluorescence-activated cell sorter (FACS) analysis. Human CD14-positive cells were separated from peripheral blood mononuclear cells (PBMCs) as described previously (36). HL60-MAG and HL60-ct cells were transfectants stably expressing human MAG and an empty vector, respectively, and were described previously (40). The recombinant virus YK333 expressing enhanced green fluorescent protein (EGFP) was described previously (43). YK333 grows as well as wild-type HSV-1(F) in cell cultures, and only YK333-infected cells express EGFP (43).

Cell fusion assay. pPEP98-gB, pPEP99-gD, pPEP101-gL, and pPEP100-gH express HSV-1 gB, gD, gL, and gH, respectively, and were described previously (31). pPEP98-gB carrying the gB-T53A, gB-T480A, or gB-T53/480A mutation was constructed as described previously (49). PILRα and nectin-1 expression vectors were constructed by cloning the cDNA of PILRα or nectin-1, respectively, into pcDNA3.1. CHO-K1 cells were cotransfected with plasmids encoding T7 RNA polymerase (pCAGT7 [30]) and PILRα or nectin-1 (target cells). CHO cells were transfected with a plasmid carrying the firefly luciferase gene under the control of the T7 promoter (pT7EMCLuc [30]), gD, gH, gL, and wild-type or mutant gB (effector cells). At 24 h posttransfection, the effector and target cells were harvested and were cocultured at a 1:1 ratio in 96-well plates for 18 h. Thereafter, luciferase activity was measured by using a luminometer (Berthold System).

Generation of recombinant viruses. To construct pCRxgBT53A, the NotI-AatII fragment of a plasmid containing HSV-1 gB with the gB-T53A mutation (49) was substituted for the NotI-AatII fragment of pCRxgB (36). To generate YK703 with the gB-T53A mutation (Fig. 1), nearly confluent Vero cells in a 24-well plate were transfected with 1 μg pCRxgBT53A with Lipofectamine (Invitrogen). At 24 h posttransfection, the cells were superinfected with the gB deletion mutant virus YK701 at a multiplicity of infection (MOI) of 5, and infection proceeded for 48 h. The infected cells were then harvested and subjected to freeze-thawing and sonication. The cell lysates were diluted and inoculated on Vero cells. Plaques were isolated and screened for the T53A amino acid substitution in gB by sequencing the DNA fragment amplified by PCR from viral DNA extracted from mature cytoplasmic virions. YK704 with the gB-T53A mutation and expressing EGFP (gB-T53A/EGFP) (Fig. 1) was constructed by the coinfection of Vero cells with YK703 and YK333 as described previously (41). Plaques were screened for fluorescence by inverted fluorescence microscopy (Olympus IX71) and for the T53A amino acid substitution as described above. To generate YK705, in which the T53A substitution in gB of YK704 had been repaired (gB-TA-repair/EGFP) (Fig. 1), rabbit skin cells were cotransfected with YK704 DNA and pCRxgB by the calcium phosphate precipitation technique as described previously (21). Viral DNAs were extracted from infected cells and purified on 5 to 20% potassium acetate gradients as described previously (42). Plaques were screened for the gB wild-type sequence as described above. To generate YK706, which carried a T480A mutation in gB (gB-T480A), the two-step Red-mediated mutagenesis procedure was carried out by using *Escherichia coli* GS1783 containing pYEbac102 as described previously (18, 20), except with primers 5′-ACACCTCCGAGAGCAGAGCCGCAAGCCCCCAA ACCCCGCGCCCCCGCCGCCCGGGGCCAGAGGATGACGACGATAAG TAGGG-3′ and 5′-GCTCCACGGACGCGTTGGCGCTGGCCCCGGGCGG CGGGGGCGCGGGGTTTGGGGGCTTGCCAACCAATTAACCAATTCTG ATTAG-3′ (Fig. 1). To generate YK707, which carried the gB-T53/480A mutation, the two-step Red-mediated mutagenesis procedure was carried out by using *E. coli* GS1783 containing pYEbac102 as described above, except with *E. coli* containing the YK706 genome and primers 5′-GTCGCGGCCGCGACCC AGGCGGCGAACGGGGGACCTGCCGCTCCGGCGCCGCCCGCCCCTA GGATGACGACGATAAGTAGGG-3′ and 5′-TCCCCCGTTGGGGCGGGG CCAGGGGCGGGCGGCGCCGGAGCGGCAGGTCCCCCGTTCGCCCAA CCAATTAACCAATTCTGATTAG-3′. YK708 with the gB-T53/480A mutation and expressing EGFP (gB-T53/480A/EGFP) (Fig. 1) was constructed by the coinfection of Vero cells with YK707 and YK333 as described above. To generate YK709, in which the gB-T53/480A mutation of YK708 had been repaired (gB-TATA-repair/EGFP) (Fig. 1), the procedure to generate YK705 was used, except with YK708 DNA. The genotype of each recombinant virus was confirmed by sequencing (data not shown).

Virus entry assay. RPMI 1640 medium, advanced RPMI 1640 medium, and Ham's F-12 medium supplemented with 1% fetal calf serum (FCS) were used for virus infection of IC21 cells, HL60 cells, CD14-positive PBMCs, and various types of CHO cells, respectively. Cells were infected with YK333, YK704, YK705, YK708, or YK709 at an MOI of 5, followed by centrifugation at 1,100 × *g* for 2 h at 32°C. For infection of Vero and HEL cells, Medium 199 supple-

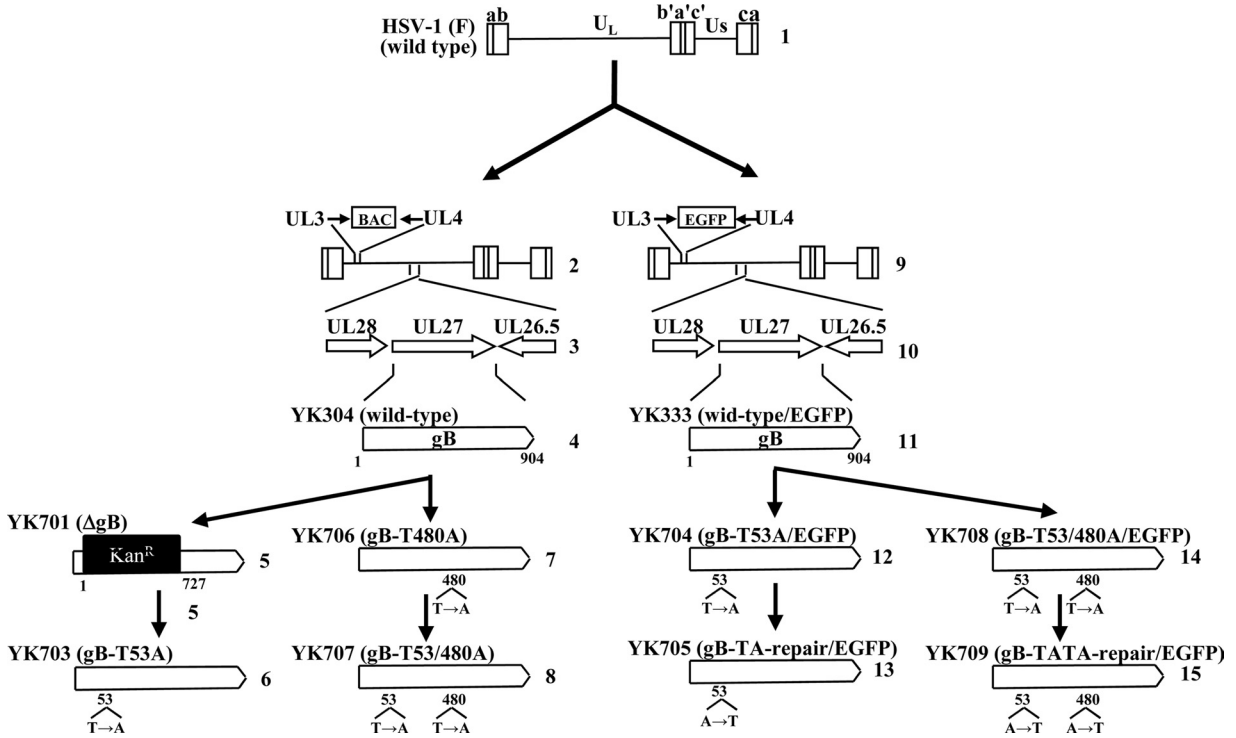

FIG. 1. Construction of recombinant viruses. (Diagram 1) Linear representation of the HSV-1(F) genome. (Diagrams 2 and 9) Linear representations of the genomes of YK304 and YK333, respectively. The YK304 and YK333 genomes contain a bacmid (BAC) or EGFP expression cassette, respectively, in the intergenic region between UL3 and UL4. (Diagrams 3 and 10) Domains encoding the UL26.5, UL27 (gB), and UL28 open reading frames. (Diagrams 4 and 11) The UL27 gene product (gB). (Diagrams 5 to 8 and 12 to 15) Schematic diagrams of the UL27 gene product (gB) in the recombinant viruses YK701, YK703, YK706, YK707, YK704, YK705, YK708, and YK709, respectively.

mented with 1% FCS was used, and virus adsorption was performed without centrifugation. At 10 h (HL60, CHO, Vero, Colon 26, and HEL cells) or 14 h (IC21 and CD14-positive PBMCs) postinfection, cells were analyzed by fluorescence microscopy, or EGFP expression was analyzed by using a FACSCalibur instrument with Cell Quest software (Becton Dickinson). In experiments with CHO-m3OST cells, HSV-1-infected cells were fixed with paraformaldehyde, permeabilized with 0.1% Triton X-100, stained with mouse monoclonal antibody to ICP4 (H640), and analyzed by using a FACSCalibur instrument.

Antibodies. Mouse monoclonal antibodies to human (M4) and mouse (153) PILRα were prepared as described previously (36). Mouse monoclonal antibodies to gB (1105) and ICP4 (H640) were purchased from the Goodwin Institute. Phycoerythrin-conjugated anti-human CD8α mouse monoclonal antibody was purchased from eBioscience.

Immunofluorescence. Immunofluorescence was performed as described previously (20).

Purification of virions. Virions were purified as described previously (20).

Animal studies. Female ICR mice were purchased from Charles River. For intracerebral infection, 3-week-old female mice were infected with various doses of each of the indicated viruses as described previously (35). Mice were monitored daily, and mortality from 1 to 28 days postinfection was attributed to the inoculated viruses. For corneal infections, 5-week-old female mice were lightly scarified on their corneas with a 27-gauge needle, the tear film was blotted, and a 3-μl drop containing 1×10^6 PFU of each of the indicated viruses was applied onto the eye as described previously (2, 35). To determine viral titers in tear films, tear film samples were collected from both eyes by using a single cotton-tipped applicator. The cotton tip was transferred into 500 μl medium 199 supplemented with 1% FCS and frozen at −80°C. Frozen samples were later thawed and thoroughly mixed, and infectious virus was quantitated by standard plaque assays on Vero cells. The total PFU per cotton tip was determined and divided by 2 to calculate the approximate viral titer per eye. Mice were monitored daily for mortality, and the clinical severity of keratitis of individually scored mice was recorded as described previously (32, 35). The clinical scoring system was as follows: 0, normal cornea; 1, mild haze; 2, moderate haze with the iris visible; 3, severe haze with the iris not visible; 4, severe haze with corneal ulcer; 5, corneal rupture. All animal studies were carried out with the approval of the Ethical Committee for Animal Experimentation at the University of Tokyo.

RESULTS

Role of the potential O-glycosylation sites of gB in PILRα-dependent membrane fusion. To examine the role of HSV-1 gB Thr-53 and Thr-480, which are required for binding to PILRα as well as the acquisition of the principal O-glycans on gB (49), in PILRα-dependent membrane fusion, we performed a cell-cell fusion assay to measure HSV-1-induced membrane fusion dependent on the transient expression of HSV-1 envelope glycoproteins (gB, gD, gH, and gL) (46). As shown in Fig. 2A, CHO cells transfected with wild-type gB showed cell fusion with PILRα-transfected CHO cells. On the other hand, CHO cells transfected with a T53A mutant gB showed apparently reduced fusion activity compared to that of cells transfected with wild-type gB. Although CHO cells transfected with a T480A mutant gB showed cell fusion with PILRα-transfected cells with an efficiency comparable to that of cells transfected with wild-type gB, cells transfected with a T53A T480A double mutant gB did not show detectable cell fusion. The fusion activity obtained with each of the gB mutants correlated well with the previously reported binding of these mutants to PILRα (49). In contrast, the T53A and T480A single mutant gB and T53A T480A double mutant gB showed cell fusion with nectin-1-transfected CHO cells at the same level as that with wild-type gB (Fig. 2B). These results indicated that Thr-53

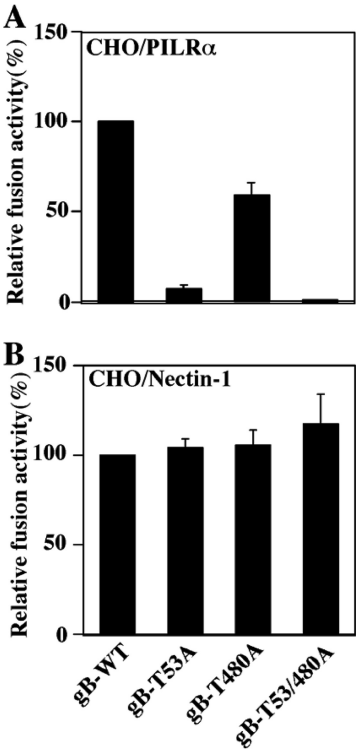

FIG. 2. Effect of the T53A and/or the T480A mutation in gB on cell-cell fusion. CHO cells were transfected with wild-type gB or mutant gB as well as other HSV-1 glycoproteins (gD, gH, and gL) and luciferase driven by a T7 promoter (effector cells) and were cocultured with CHO cells transfected with PILRα (A) or nectin-1 (B) and T7 polymerase (target cells). After 18 h of incubation, luciferase activity was measured. Cell fusion activities relative to that of wild-type gB are shown. These results are the averages and standard errors of data from three independent experiments.

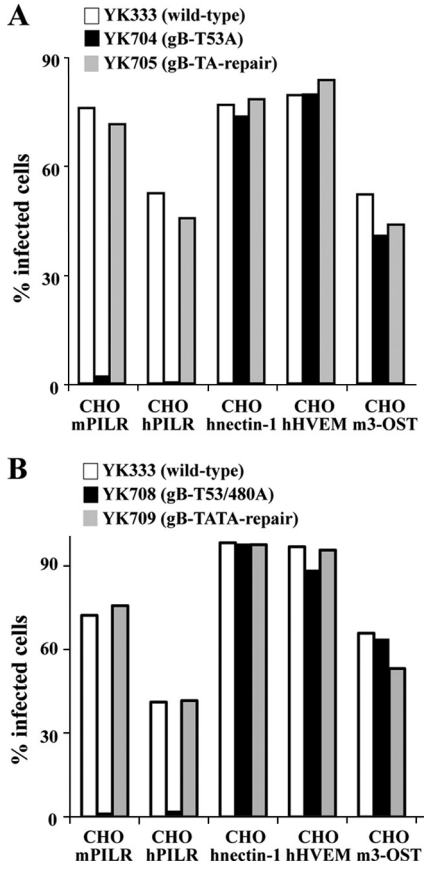

FIG. 3. Effect of the T53A and T53/480A mutations in gB on viral entry into CHO cells expressing viral receptors. CHO transformants were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), or YK705 (gB-TA-repair/EGFP) (A) or with YK333 (wild-type/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) (B). At 10 h postinfection, live infected CHO-mPILR, CHO-hPILR, CHO-hNectin-1, and CHO-hHVEM cells were examined by flow cytometry, and infected CHO-m3-OST cells were fixed, permeabilized, stained with anti-ICP4 antibody, and examined by flow cytometry.

alone and both Thr-53 and Thr-480 in gB were critical for PILRα-dependent membrane fusion.

Effect of the gB-T53A and gB-T53/480A mutations on PILRα-dependent viral entry. To examine the role of HSV-1 gB Thr-53 alone or of both gB Thr-53 and Thr-480 in natural infections, we constructed the recombinant viruses YK704 (gB-T53A/EGFP) and YK708 (gB-T53/480A/EGFP) with the gB-T53A and gB-T53/480A mutations, respectively, and expressing EGFP (Fig. 1). In addition, we constructed YK705 (gB-TA-repair/EGFP) and YK709 (gB-TATA-repair/EGFP), in which the gB-T53A mutation in YK704 and the gB-T53/480A mutation in YK708, respectively, were repaired (Fig. 1). CHO-mPILRα, CHO-hPILRα, CHO-hNectin-1, CHO-hHVEM, CHO-m3OST, and CHO-neo cells were transformants stably expressing human PILRα, mouse PILRα, human nectin-1, mouse heparan sulfate 3-*O*-ST 3B1, human HVEM, and empty vector, respectively. Each CHO cell type was infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), YK705 (gB-TA-repair/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP), and infection was analyzed by monitoring the EGFP fluorescence by flow cytometry (Fig. 3). As shown in Fig. 3, CHO-hNectin-1, CHO-hHVEM, and CHO-m3OST cells, which express known gD receptors, were efficiently infected by all five viruses. In contrast, CHO-mPILRα and CHO-hPILRα cells were efficiently infected by YK333 (wild-type/EGFP), YK705 (gB-repair/EGFP), and YK709 (gB-TATA-repair/EGFP) but not by YK704 (gB-T53A/EGFP) or YK708 (gB-T53/480A/EGFP). Furthermore, in HL60 cells overexpressing another gB receptor, MAG, the efficiency of YK704 (gB-T53A/EGFP) entry was similar to those of YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP), and the efficiency of YK708 (gB-T53/480A/EGFP) entry was similar to those of YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP) (Fig. 4). These results indicated that Thr-53 alone or both Thr-53 and Thr-480 in gB were essential for PILRα-dependent viral entry in CHO cells and that the loss of these sites by mutation had no effect on viral entry via known gD receptors and another gB receptor, MAG.

We next investigated the effect of the gB T53A and gB-T53/480A mutations on viral entry into normal cell lines and primary human cells. IC21 cells and primary CD14-positive human monocytes express PILRα endogenously (36, 37), while Vero and HEL cells do not (data not shown). Furthermore, we

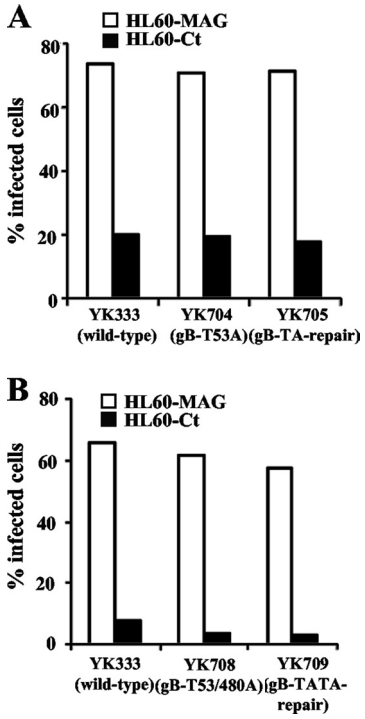

FIG. 4. Effect of the T53A and T53/480A mutations in gB on viral entry into HL60 cells expressing MAG. HL60-MAG and HL60-ct cells were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), or YK705 (gB-TA-repair/EGFP) (A) or with YK333 (wild-type/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) (B) at an MOI of 5. At 10 h postinfection, live infected cells were examined by flow cytometry.

previously demonstrated that HSV-1 infection of IC21 cells and CD14-positive human monocytes is dependent on the presence of cell surface PILRα (1, 36). Therefore, for these studies, Vero, HEL, and IC21 cells and CD14-positive human primary monocytes separated from peripheral blood mononuclear cells (PBMCs) were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), YK705 (gB-TA-repair/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) and analyzed for EGFP fluorescence by flow cytometry at 10 h (Vero and HEL cells) or 14 h (IC21 cells and CD14-positive PBMCs) postinfection. As shown in Fig. 5A and B, the ability of YK704 (gB-T53A/EGFP) to enter PILRα-positive cells was significantly impaired compared to those of YK333 (wild-type/EGFP) and YK705 (gB-repair/EGFP). Similar results were also obtained with YK708 (gB-T53/480A/EGFP) and YK709 (gB-TATA-repair/EGFP), although IC21 cells appeared to be less susceptible to YK705 (gB-T53/480A/EGFP) than to YK704 (gB-T53A/EGFP) (Fig. 5E and F). These results indicated that gB Thr-53 alone and both Thr-53 and Thr-480 were critical for PILRα-dependent viral entry into cells, both in CHO cells overexpressing PILRα and in normal cells expressing endogenous PILRα.

In PILRα-negative Vero (Fig. 5C and G), Colon 26 (Fig. 5D and H), and HEL (data not shown) cells, YK704 (gB-T53A/EGFP) entry was similar to those of YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP), and YK708 (gB-T53/480A/EGFP) entry was similar to those of YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP). Furthermore, virus growth curves of Vero cells infected at an MOI of 5 or 0.01 with YK704 (gB-T53A/EGFP) were essentially identical to those of YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP), and the growth curves of YK708 (gB-T53/480A/EGFP) were essentially identical to those of YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP) (Fig. 6). Consistent with these results, in Vero and HEL cells infected with each of these viruses at MOIs of 0.01 and 5, at 24 h postinfection the intracellular and extracellular virus yields of YK704 (gB-T53A/EGFP) were similar to those of YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP), and the yield of YK708 (gB-T53/480A/EGFP) was similar to those of YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP) (data not shown). We also note that (i) the sizes of plaques produced on Vero cells (Fig. 7A), Colon 26 cells (Fig. 7B), and HEL cells (data not shown) by YK704 (gB-T53A/EGFP) were similar to those produced by YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP), and the size produced by YK708 (gB-T53/480A/EGFP) was similar to those produced by YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP) (Fig. 7C and D); (ii) the intracellular localization of the gB T53A and T53/480A mutants in Vero cells infected with YK704 (gB-T53A/EGFP) and YK708 (gB-T53/480A/EGFP), respectively, as determined by immunofluorescence, was similar to those of gB in cells infected with YK333 (wild-type/EGFP) and the repaired viruses YK705 (gB-TA-repair/EGFP) and YK709 (gB-TATA-repair/EGFP), respectively (data not shown); (iii) in infected Vero cells, the efficiency of packaging of T53A mutant gB into YK704 (gB-T53A/EGFP) virions was similar to those into YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP) virions, and the efficiency of packaging of T53/480A gB into YK708 (gB-T53/480A/EGFP) virions was similar to those into YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP) virions (data not shown); and (iv) the effect of heparin on YK704 (gB-T53A/EGFP) adsorption was identical to its effects on YK333 (wild-type/EGFP) and the repaired virus YK705 (gB-TA-repair/EGFP), and its effect on YK708 (gB-T53/480A/EGFP) adsorption was identical to its effects on YK333 (wild-type/EGFP) and the repaired virus YK709 (gB-TATA-repair/EGFP), indicating that the gB-T53A and gB-T53/480A mutations had no effect on virus binding to the cell surface via heparan sulfate (Fig. 8). Taken together, these results indicated that YK704 (gB-T53A/EGFP) and YK708 (gB-T53/480A/EGFP) exhibited a phenotype almost identical to those of YK333 (wild-type/EGFP) and their repaired viruses in PILRα-negative cells.

Effect of gB-T53A and gB-T53/480A mutations on viral replication and pathogenesis in mice. To determine the effect of the gB-T53A and gB-T53/480A mutations on viral replication and pathogenesis *in vivo*, we studied two murine models of HSV-1 infection. In experimental animal models of HSV-1 infection, viral invasion of the central nervous system (CNS) from peripheral sites (neuroinvasiveness) and CNS destruction caused by viral replication (neurotoxicity) are semi-independent indicators of viral virulence and are tested by peripheral and intracerebral inoculation, respectively (47). In the first

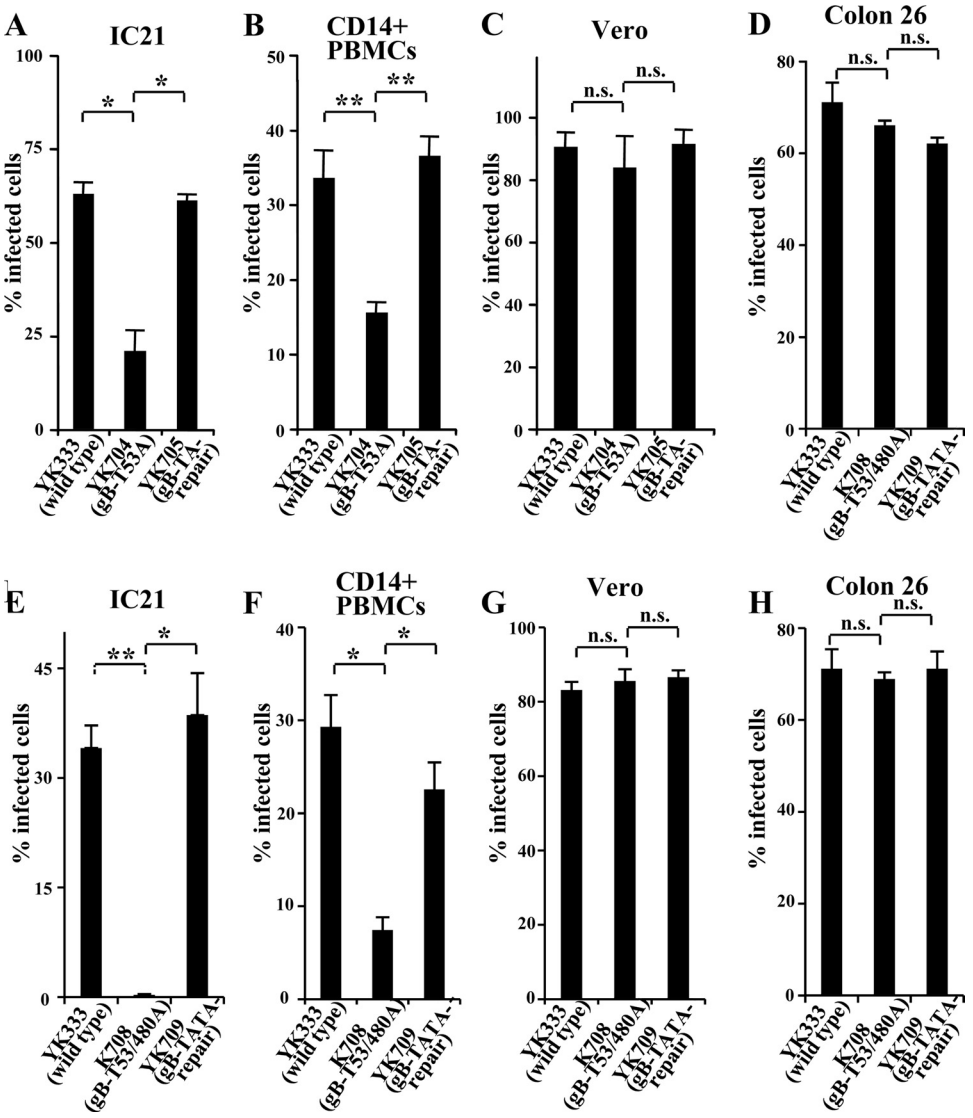

FIG. 5. Effect of the T53A and T53/480A mutations in gB on viral entry in normal cell lines. IC21 cells (A and E), primary CD14-positive PBMCs (B and F), Vero cells (C and G), and Colon 26 cells (D and H) were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), or YK705 (gB-repair/EGFP) (A to D) or with YK333 (wild-type/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) (E to H), and the fraction of infected cells was determined by flow cytometry. These results are the averages and standard errors of data from three independent experiments. The statistical differences between the proportions of IC21 and PBMCs infected with YK333 (wild-type/EGFP) or YK705 (gB-TA-repair/EGFP) and cells infected with YK704 (gB-T53A/EGFP) were significant in A and B (*, $P < 0.05$; **, $P < 0.001$), but the difference was not significant (n.s.) for Vero and Colon 26 cells (C and D), as noted. The statistical differences between the proportions of IC21 and PBMCs infected with YK333 (wild-type/EGFP) or YK708 (gB-TATA-repair/EGFP) and cells infected with YK708 (gB-T53/480A/EGFP) were significant in E and F (*, $P < 0.05$; **, $P < 0.001$), but the difference was not significant for Vero and Colon 26 cells (G and H), as noted.

series of animal model experiments, we assayed the neurotoxicity of the mutant viruses described above by the intracerebral inoculation of mice with various doses of YK704 (gB-T53A/EGFP) and YK705 (gB-repair/EGFP) and monitoring mortality daily. The survival curves for mice infected with various doses of YK704 (gB-T53A/EGFP) were similar to those for mice infected with YK705 (gB-repair/EGFP) (Fig. 9A to D). Similarly, the survival curves for mice infected with 10^3 PFU YK708 (gB-T53/480A/EGFP) were similar to those for mice infected with YK709 (gB-TATA-repair) (Fig. 9E). These results indicated that the gB-T53A and gB-T53/480A mutations had no significant effect on neurotoxicity in the murine model.

In the second series of animal model experiments, we employed the murine model for HSK to study neuroinvasiveness and pathogenesis (2, 32). Mice were inoculated ocularly with YK704 (gB-T53A/EGFP), YK705 (gB-repair/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) and observed daily for mortality and the development of HSK. In addition, to examine viral replication at the infection site, tear film samples were collected at the times indicated postinfection, and viral titers were determined. As shown in Fig. 10A and B and 11A and B, mice infected with YK704 (gB-T53A/EGFP) or YK708 (gB-T53/480A/EGFP) exhibited significantly reduced HSK severity and mortality compared to those of mice

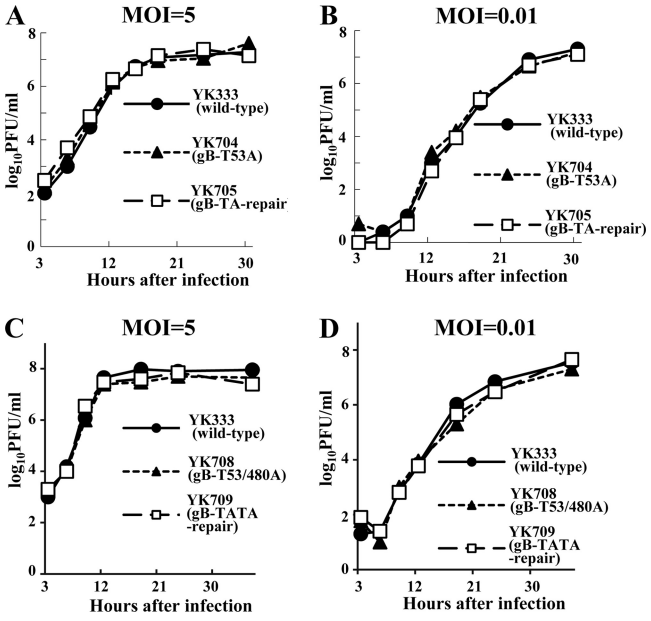

FIG. 6. Effect of the T53A and T53/480A mutations in gB on viral replication in Vero cells. Vero cells were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), or YK705 (gB-TA-repair/EGFP) (A and B) or with YK333 (wild-type/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) (C and D) at an MOI of 5 (A and C) or 0.01 (B and D). At the times indicated postinfection, total virus from cell culture supernatants and infected cells was assayed on Vero cells.

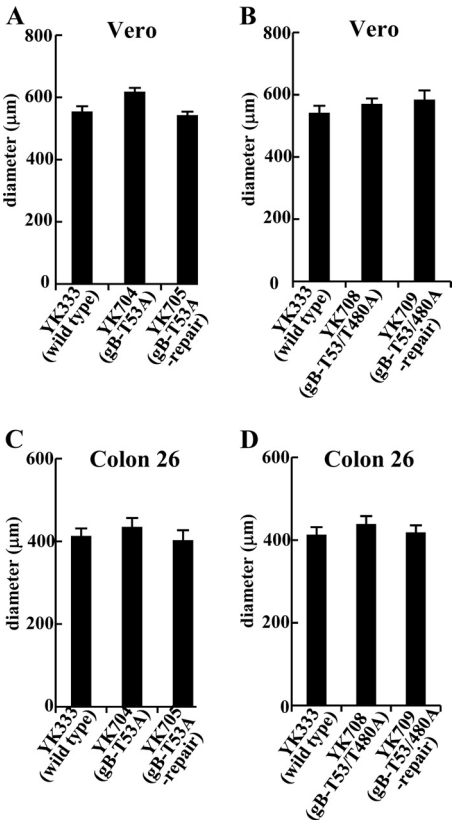

FIG. 7. Effect of the T53A and T53/480A mutations in gB on plaque formation on Vero and Colon 26 cells. Vero (A and B) or Colon 26 (C and D) cells were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), or YK705 (gB-repair/EGFP) (A and C) or with YK333 (wild-type/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) (B and D) under plaque assay conditions for 2 days. The results are the average diameters and standard errors of 30 (Vero cells) or 20 (Colon 26 cells) single plaques per each recombinant virus.

infected with YK705 (gB-TA-repair/EGFP) or YK709 (gB-TATA-repair/EGFP), respectively. In this animal model, mortality is a measure of CNS invasion from peripheral sites (i.e., neuroinvasiveness) (8, 26). In addition, YK704 (gB-T53A/EGFP) and YK708 (gB-T53/480A/EGFP) replicated significantly less efficiently in the tear films of these infected mice, with titers approximately 10-fold lower than those of YK705 (gB-TA-repair/EGFP) and YK709 (gB-TATA-repair/EGFP), respectively (Fig. 10C and 11C). These results indicated that gB Thr-53 alone and both gB Thr-53 and Thr-480 were required for efficient viral replication, the development of HSK, and neuroinvasiveness in the murine corneal model.

DISCUSSION

In the present study, we have further analyzed the role of HSV-1 gB's two threonines (Thr-53 and Thr-480), which we previously identified as sites required both for the binding to PILRα and for the addition of the principal O-glycans to gB (49), in PILRα-dependent viral entry. Since an enveloped virus requires an entry receptor to mediate membrane fusion, followed by binding to a viral ligand, we first examined whether an alanine substitution(s) in the gB threonines affects membrane fusion dependent on HSV-1 glycoproteins. In agreement with previously observed reductions in binding to PILRα due to the gB-T53A and gB-T53/480A mutations, these mutations significantly reduced membrane fusion activity in CHO cells expressing PILRα. Second, we generated recombinant viruses carrying the gB-T53A and gB-T53/480A mutations and demonstrated that these recombinant viruses were not able to enter CHO cells expressing PILRα. The impairment of membrane fusion and viral entry observed with these mutants was dependent on the presence of PILRα, based on the observations that the mutants exhibited wild-type membrane fusion and viral entry in CHO cells expressing a gD receptor(s). These results support the hypothesis that the two threonines in gB are in fact critical for PILRα-dependent viral entry. Since the gB-T53/480A mutation inhibited the principal O-glycosylation sites on gB (49), the results also suggest that the O-glycosylation of gB plays an important role in PILRα-dependent viral entry. However, it is possible that the defect in membrane fusion and viral entry observed with the gB mutants might be due to the steric hindrance of gB caused by the amino acid replacement(s) rather than being due to the prevention of the O-glycosylation of gB. Although we cannot completely exclude this possibility, it seems unlikely based on the following observations. (i) We have presented data that, except for PILRα-dependent membrane fusion and viral entry, the gB-T53A and the gB-T53/480A mutations had no effect on various aspects of viral replication associated with known gB functions, including viral entry via known gD receptors and MAG (another HSV-1 gB receptor), viral attachment to heparan sulfate, viral cell-to-cell

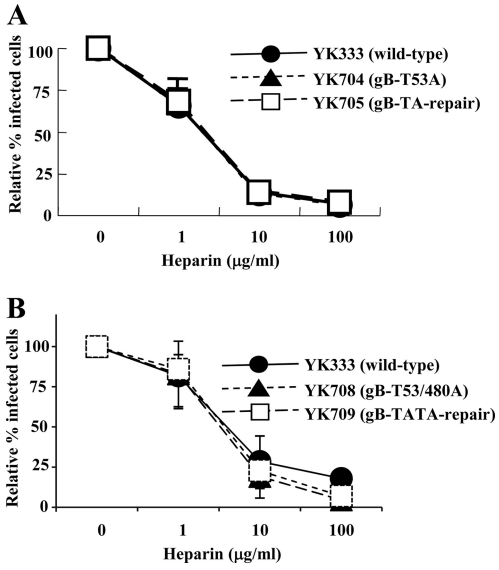

FIG. 8. Effect of heparin on recombinant virus adsorption. Subconfluent Vero cells were infected with YK333 (wild-type/EGFP), YK704 (gB-T53A/EGFP), or YK705 (gB-TA-repair/EGFP) (A) or with YK333 (wild-type/EGFP), YK708 (gB-T53/480A/EGFP), or YK709 (gB-TATA-repair/EGFP) (B) at an MOI of 0.1 at 4°C. After 2 h of adsorption at 4°C, cells were washed three times at 4°C with medium 199 supplemented with 1% FCS and 0, 1, 10, or 100 μg heparin/ml. Medium 199 supplemented with 1% FCS, at 37°C, was then added, and the cells were placed into a 37°C incubator. At 10 h postinfection, EGFP-positive cells were determined by using a FACSCalibur instrument. These results are the averages and standard errors of data from three independent experiments, calculated relative to the sample with no heparin.

spread as observed by plaque formation, the secretion of extracellular viruses, viral replication in PILRα-negative cells, the intracellular localization of gB in infected cells, and the packaging of gB into virions. If the amino acid replacement(s) causes a significant steric hindrance of gB, one or some of these aspects might also be affected. (ii) Although the structure of HSV-1 gB has been reported, the regions around Thr-53 and Thr-480 have not yet been resolved (15). This suggested that structures around these regions are flexible and disordered. It is known that disordered regions of proteins are intrinsically unstructured and unfolded in the native structure (53). Therefore, it is unlikely that point mutations at flexible regions significantly affect the core structure of gB.

Important *in vivo* cellular targets of HSV-1 are epithelial cells at the initial site of infection and neurons for the establishment of latent infection (34). PILRα is expressed in myeloid cells and certain types of cells in neural tissues (13, 29, 36). However, the biological significance of PILRα in viral replication and pathogenesis *in vivo* remains unclear, whereas evidence that PILRα can act as an HSV-1 receptor in cell cultures is accumulating (1, 11, 12, 36). In the present study, we tested recombinant viruses carrying the gB-T53A and the gB-T53/480A mutations in the murine HSK model and demonstrated that the mutations in gB significantly reduced viral replication in eyes, the development of HSK, and neuroinvasiveness in mice. As described above, these recombinant viruses exhibited phenotypes identical to those of wild-type or

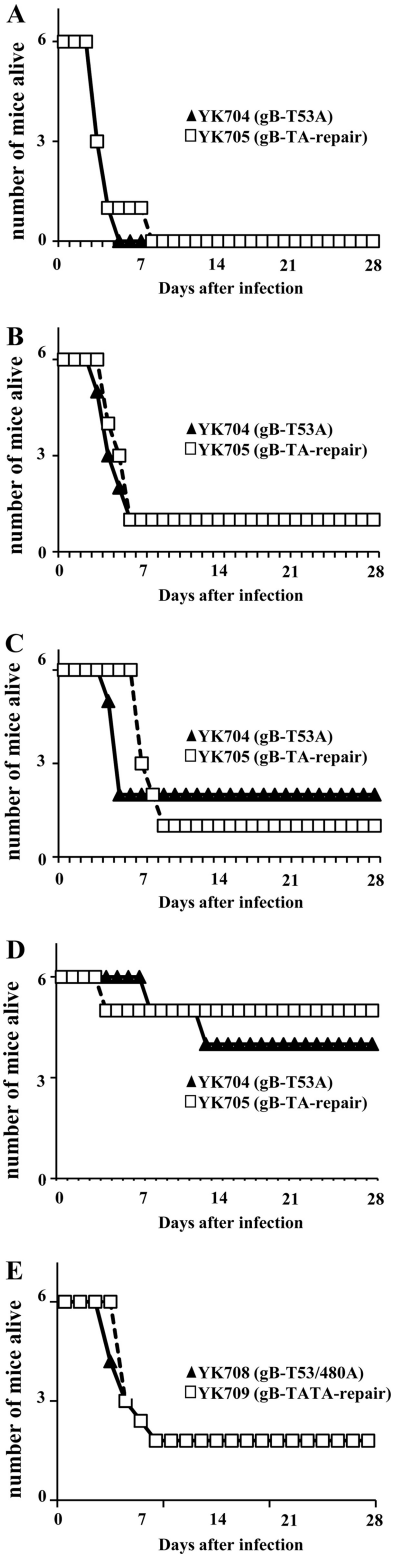

FIG. 9. Effect of the T53A and T53/480A mutations in gB on mortality of mice following intracerebral infection. Groups of six (A to D) or 10 (E) 3-week-old female ICR mice were infected with 10^4 PFU (A), 10^3 PFU (B), 10^2 PFU (C), or 10 PFU (D) YK704 (gB-T53A/EGFP) or YK705 (gB-TA-repair/EGFP) or with 10^3 PFU (E) YK708 (gB-T53/480A/EGFP) or YK709 (gB-TATA-repair/EGFP) intracerebrally and monitored for mortality daily for 28 days.

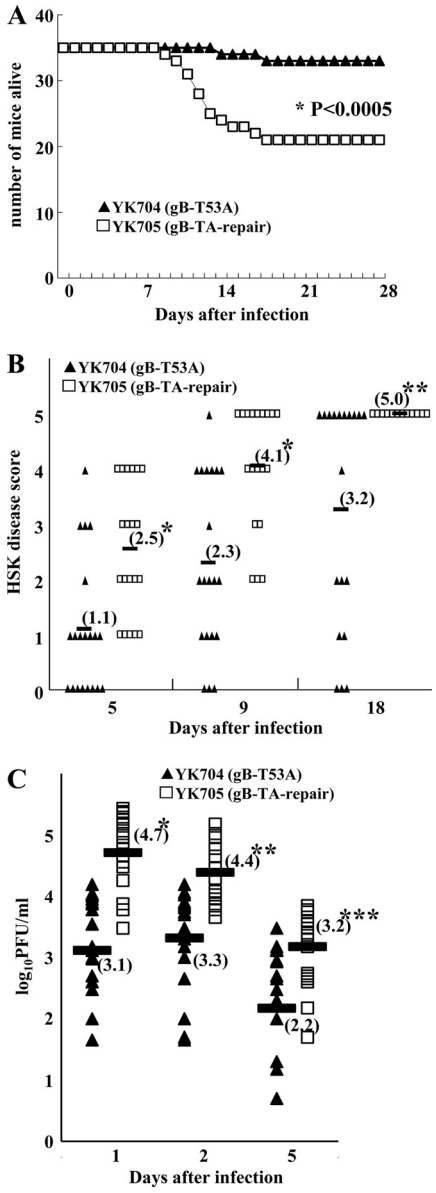

FIG. 10. Effect of the T53 mutation in gB on viral pathogenesis in mice following corneal infection. (A) Groups of 5, 10, or 20 5-week-old female ICR mice were infected with YK704 (gB-T53A/EGFP) or YK705 (gB-TA-repair/EGFP) by corneal scarification and monitored daily, for at least 28 days, for mortality. The results from three independent experiments (one with 5 mice, one with 10 mice, and one with 20 mice) were combined and are shown. The statistical difference between mice infected with YK704 (gB-T53A/EGFP) and those infected with YK705 (gB-TA-repair/EGFP) was significant, as noted (*, $P < 0.0005$). (B) For the group of 20 mice in the experiment described above (A), each mouse was scored daily for the severity of HSK disease. The HSK scores recorded 5, 9, and 18 days postinfection are shown. Each data point is the HSK score from one mouse. The horizontal bars and numbers in parentheses indicate the averages for each group. The statistical difference between HSK scores for mice infected with YK704 (gB-T53A/EGFP) and those for mice infected with YK705 (gB-TA-repair/EGFP) was significant, as noted (*, $P < 0.005$; **, $P < 0.05$). (C) For the mice in B, viral titers in the tear film of infected mice at 1, 2, and 5 days postinfection were determined by standard plaque assays. Each data point represents the titer in the tear film of one mouse. The horizontal bars and numbers in parentheses indicate the averages for each group. The statistical difference between viral titers in mice infected with YK704 (gB-T53A/EGFP) and those in mice infected with YK705 (gB-TA-repair/EGFP) was significant, as noted (*, $P < 0.0000001$; **, $P < 0.00001$; ***, $P < 0.0005$).

repaired mutant viruses except that the mutant viruses had a defect in PILRα-dependent viral entry. Therefore, it is likely that PILRα-dependent viral entry, which requires gB O-glycosylation, plays a significant role in viral replication, pathogenesis, and neuroinvasiveness *in vivo*. However, as is usual with such mutational analyses, we cannot completely eliminate the possibility that T53A and T53/480A mutations in gB had an effect on a function(s) of gB other than those tested in this study. Further analyses will be necessary to define the role(s) of PILRα in HSV-1 entry in viral infection and diseases *in vivo*. Such experiments might include the use of PILRα-deficient knockout mice, similar to the use of mice with nectin-1 and/or HVEM knocked out to demonstrate alternative roles of these receptors in viral replication and disease (24, 44). By using such genetically engineered mice, it might be possible to evaluate directly the contribution of specific molecules to viral infection *in vivo*. However, the generation of mice with a disruption of the PILRα gene has not yet been reported. Furthermore, if a specific mouse receptor plays a critical role(s) in the regulation of viral infection *in vivo* other than viral entry (e.g., in the immune response to viral infection), it would be difficult to determine definitely whether an experimental result for mice with a disrupted receptor gene might be due to an effect on viral entry via the receptor or an effect on some other function of the receptor. Therefore, the use of viral manipulation analyses in animal models to investigate the role of a specific viral entry receptor *in vivo* without modifying any host cell functions, as reported here, may complement data from studies using genetically engineered mice, and vice versa.

At present, it is not known how PILRα contributes to HSV-1 replication and pathogenesis *in vivo*. In this study, we found (i) greater mortality in mice with peripheral (ocular) infection with YK705 (gB-TA-repair/EGFP) than in mice infected with YK704 (gB-T53A/EGFP) and greater mortality in mice with peripheral infection with YK709 (gB-TATA-repair/EGFP) than in mice infected with YK708 (gB-T53/480A/EGFP); (ii) similar mortalities in mice with intracerebral infections with YK704, YK705, YK708, and YK709; and (iii) similar growth properties in cell cultures infected with these viruses. These results suggest that pathogenesis following PILRα-dependent viral entry has a significant immunological component, as was previously reported (5, 10). Consistent with this suggestion, PILRα is expressed in immune system cells (such as macrophages and dendritic cells) and regulates the function of these cell populations (13). Therefore, it is possible that HSV-1 infection of PILRα-positive immune system cells (such as macrophages and dendritic cells) modulates the activity of these cells, leading to an attenuation of innate and adaptive immune responses against HSV-1 infection. In support of this hypothesis, it was reported previously that HSV-1 can infect macrophages and dendritic cells (4, 27) and that macrophages and dendritic cells infiltrate specific tissues 1 day after HSV infection in mice (6, 25), and it was suggested that the antiviral activities of macrophage cells may be important mediators of innate HSV-1 resistance (10, 22). These potential immunological effects may regulate viral replication *in vivo*, and the reduced replication capacities of YK704 (gB-T53A/EGFP) and YK708 (gB-T53/480A/EGFP) compared to those of the repaired viruses may account for their lower pathogenicity in HSK and neuroinvasiveness.

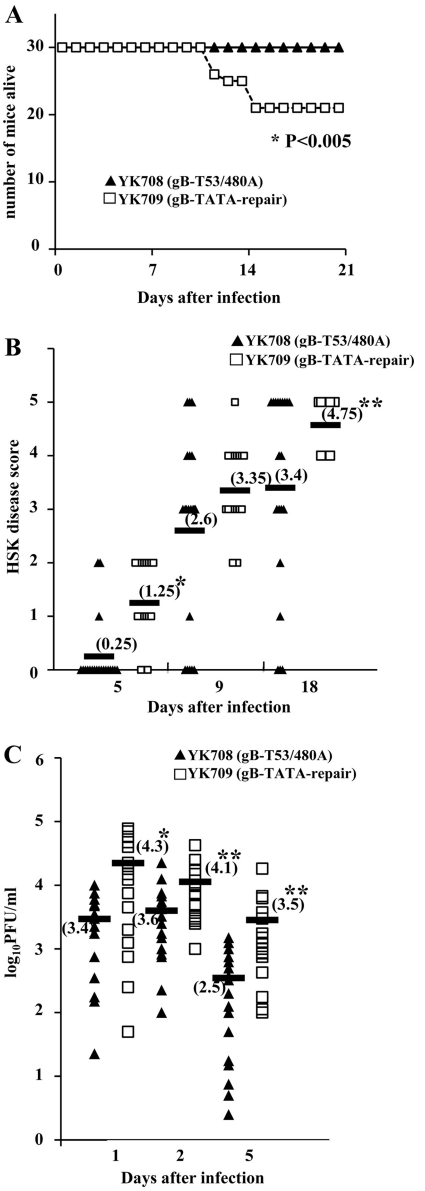

FIG. 11. Effect of the T53/480A mutation in gB on viral pathogenesis in mice following corneal infection. (A) Groups of 10 or 20 5-week-old female ICR mice were infected with YK708 (gB-T53/480A/EGFP) or YK709 (gB-TATA-repair/EGFP) by corneal scarification and monitored daily, for at least 28 days, for mortality. The results from two independent experiments (one with 10 mice and two with 20 mice each) were combined and are shown. The statistical difference between mice infected with YK708 (gB-T53/480A/EGFP) and those infected with YK709 (gB-TATA-repair/EGFP) was significant, as noted (*, $P < 0.005$). (B) For one of the groups of 20 mice in the experiment described above (A), each mouse was scored daily for the severity of HSK disease. The HSK scores recorded 5, 7, and 18 days postinfection are shown. Each data point is the HSK score from one mouse. The horizontal bars and numbers in parentheses indicate the averages for each group. The statistical difference between HSK scores of mice infected with YK708 (gB-T53/480A/EGFP) and those of mice infected with YK709 (gB-TATA-repair/EGFP) was significant, as noted (*, $P < 0.0001$; **, $P < 0.05$). (C) For the mice in B, viral titers in the tear film of infected mice at 1, 2, and 5 days postinfection were determined by standard plaque assays. Each data point represents the titer in the tear film of one mouse. The horizontal bars and numbers in parentheses indicate the averages for each group. The statistical difference between viral titers in mice infected with YK708 (gB-T53/480A/EGFP) and those in mice infected with YK709 (gB-TATA-repair/EGFP) was significant, as noted (*, $P < 0.005$; **, $P < 0.01$).

In conclusion, the data presented here are the first, to our knowledge, suggesting that the ability of gB to associate with PILRα plays a role in HSV-1 replication and pathogenesis *in vivo*. Coupled with previous reports demonstrating significant roles of gD receptors in viral replication and pathogenesis in mice (24, 44), these findings support the hypothesis that cellular receptors for both gB and gD are required for efficient HSV-1 infection *in vivo* and that HSV-1 tropisms *in vivo* are determined by combinations of two different sets of entry receptors for gB and gD. The results of this study should be significant for an understanding of the molecular basis of HSV-1 pathogenesis. In addition, our data also support the hypothesis that the O-glycosylation of gB is critical for HSV-1 replication and pathogenesis *in vivo*. It was reported previously that glycans on the viral envelope often have a crucial role in enabling the efficient transmission of the pathogen and/or entry into susceptible host cells (9, 45, 48). Moreover, it was shown that the presence of glycans on enveloped viruses is also important for the evasion of host immunological surveillance (33, 52). Our observations raised the interesting possibility that the glycosylation of gB may regulate cell tropism, immune evasion from the host immune response, and the pathogenesis of HSV-1 *in vivo*.

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