

## RAPID COMMUNICATION

Downregulation of  $\beta 1$  Integrins by Ebola Virus Glycoprotein: Implication for Virus EntryAyato Takada,\*† Shinji Watanabe,† Hiroshi Ito,† Katsunori Okazaki,\* Hiroshi Kida,\* and Yoshihiro Kawaoka†‡<sup>1</sup>

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Filoviruses, including Ebola virus, are cytotoxic. To investigate the role of the Ebola virus glycoprotein (GP) in this cytopathic effect, we transiently expressed the GP in human kidney 293T cells. Expression of wild-type GP, but not the secretory form of the molecule lacking a membrane anchor, induced rounding and detachment of the cells, as did a chimeric GP containing its ectodomain and influenza virus hemagglutinin transmembrane–cytoplasmic domain. These results indicate that the GP ectodomain and its anchorage to the membrane are required for GP-induced morphologic changes in host cells. Since cell rounding and detachment could be associated with reduced levels of cell adhesion molecules, we also studied the expression of integrins, which are major molecules for adhesion to extracellular matrices, and found that the  $\beta 1$  integrin group is downregulated by the GP. This result was further extended by experiments in which anti- $\beta 1$  monoclonal antibodies or purified integrins inhibited the infectivity of vesicular stomatitis virus pseudotyped with the GP. We suggest that integrins, especially the  $\beta 1$  group, might interact with the GP and perhaps be involved in Ebola virus entry into cells. © 2000 Academic Press

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Ebola virus, a filamentous, enveloped, negative-strand RNA virus in the family *Filoviridae*, causes severe hemorrhagic fever in humans and other primates (11). Four subtypes of the virus have been identified: Zaire, Sudan, Ivory Coast, and Reston (12). The fourth gene from the 3' end of the Ebola virus nonsegmented genome encodes two glycoproteins: the primary product, termed nonstructural secretory glycoprotein (SGP), is secreted from infected cells (11), while the envelope glycoprotein (GP) is responsible for virus binding to cell surface molecules and penetration. The GP is expressed by transcriptional editing, resulting in the addition of an extra adenosine within a run of seven adenines in the coding region of the molecule (12, 15).

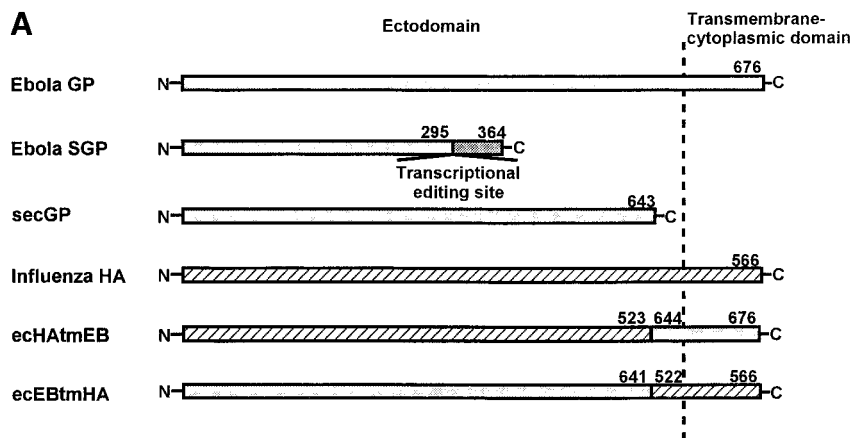
Both GP and SGP are thought to be involved in the pathogenesis of Ebola virus infection. SGP is reported to bind to neutrophils via the Fc $\gamma$  receptor and inhibit the early activation of neutrophils (16). In contrast to SGP, GP is thought to contribute to tissue tropism, since a murine retroviral vector pseudotyped with Ebola GP infected endothelial cells more efficiently than other cell types tested (16). The destruction of endothelial cells by Ebola viruses likely contributes to the hemorrhagic manifesta-

tions that characterize filovirus infections (13), although the precise mechanism(s) of this effect remains unknown. As an initial step in understanding the pathogenesis of filovirus infection, we investigated the cytopathic effects produced by Ebola virus in cultured cells (11), focusing on the pathogenic contributions of the GP and SGP.

Transient expression of GP or SGP (Fig. 1A) in 293T cells blocked the growth of transfected cells (data not shown). Expression of the GP, but not the SGP, induced morphologic rounding and detachment of the cells (Figs. 1B and 1C), suggesting the importance of membrane anchorage of this molecule for cytopathic effects. This prediction was tested with a mutant GP lacking the transmembrane–cytoplasmic domain. Since the mutant was designed to be secreted into the culture medium, it was designated secreted GP (secGP; Fig. 1A). Cross-linking analysis revealed that the secGP formed a trimer, as does authentic GP (data not shown). However, expression of the secGP did not affect cell morphology (Figs. 1B and 1C). These findings confirm a role of the transmembrane and cytoplasmic domains in the cytopathic effects of Ebola virus.

To determine which of the GP domains participates in cytopathic effects, we substituted the transmembrane–cytoplasmic domain of GP with that of influenza virus hemagglutinin (HA) and vice versa (Fig. 1A). These chi-

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**FIG. 1.** (A) Schematic diagram of mutant GPs. The secGP contains only the Ebola Zaire GP ectodomain. The ecHAtmEB contains the influenza virus (A/turkey/Ireland/1378/83; H5N8) HA (IFV HA) ectodomain (residues HA1–523) and Ebola GP transmembrane–cytoplasmic domain (residues GP644–676). The ecEBtmHA contains Zaire GP ectodomain (positions GP1–641) and HA transmembrane–cytoplasmic domain (positions HA522–566). (B) Morphologic changes induced by the expression of Ebola and influenza virus glycoproteins. Ebola Zaire and Reston GP and SGP, secGP, influenza virus HA, and chimeric protein (ecHAtmEB and ecEBtmHA) genes were cloned into a mammalian expression vector, pCAGGS/MCS (7, 10), and transfected into 293T cells. Pictures were taken 24 h posttransfection. (C) Immunologic detection of cells expressing Ebola and influenza virus glycoproteins. 293T cells were transfected with a protein expression plasmid (as described for B), fixed 24 h posttransfection, and processed for the immunologic detection of the proteins with polyclonal (Ebola glycoproteins) or monoclonal (influenza virus HA) antibodies. Note that similar percentages of cells are expressing the indicated protein.

meric mutants, the wild-type GP, and the influenza virus HA were expressed in 293T cells and the resultant cytopathologic effects were assessed (Fig. 1B). Expression of the mutant containing the Ebola GP transmembrane and cytoplasmic tail region and the HA ectodomain (ecHAtmEB) did not affect cell morphology. By contrast, the chimera containing the GP ectodomain and the other domains from the influenza HA (ecEBtmHA) induced cytopathic effects similar to those seen with wild-type GP (although to a lesser extent). Thus, the GP ectodomain may serve as a functional domain to induce morphologic changes in host cells, with membrane anchorage of the GP being a requirement for this effect.

Because of their role in cell adhesion to extracellular matrices, including fibronectin, vitronectin, and collagen (3), integrins might be involved in the cytopathic effects caused by Ebola GP. These molecules constitute a superfamily of heterodimers with noncovalently associated  $\alpha$ - and  $\beta$ -chain subunits, both of which are N-glycosylated transmembrane proteins (3). We focused on the cell surface expression of the  $\beta$ 1 group, also known as VLA receptors, since they exist ubiquitously in a wide variety of cells (3). 293T cells were transfected with a plasmid expressing the GP of the Zaire or Reston strain of Ebola virus or the influenza virus HA and analyzed by FACS for cell surface expression of integrins. We found that both the Zaire and the Reston GPs downregulated the surface expression of  $\beta$ 1 integrins, as indicated by the reduced expression of these molecules in GP-expressing (phycoerythrin-positive) cells (Fig. 2A, panels 1 and 2). By contrast, the influenza virus HA did not affect cell surface

expression of the  $\beta$ 1 integrins on the cell surface (Fig. 2A, panel 3). Similar results were obtained with the  $\alpha$ V subunit (Fig. 2A, panels 5–7), but only minimal downregulation was observed with the other  $\alpha$  molecules tested ( $\alpha$ 1  $\alpha$ 2  $\alpha$ 3,  $\alpha$ 4,  $\alpha$ 5, and  $\alpha$ 6; see results for  $\alpha$ 5 in Fig. 2A, panels 9–12, for an example). Expression of the other  $\beta$  molecules tested ( $\beta$ 2 and  $\beta$ 3) was below the detection level of our assay using 293T cells. The chimera ecEBtmHA (i.e., GP ectodomain and HA transmembrane–cytoplasmic domain), but not ecHAtmEB (HA ectodomain and GP transmembrane–cytoplasmic domain), also reduced the expression of  $\beta$ 1 integrins (Fig. 2B), although to a lesser extent than the wild-type GP. Thus, downregulation of  $\beta$ 1 integrins is likely to be associated with the cytopathic effects caused by Ebola virus.

We speculate that Ebola virus GPs and integrin molecules interact, leading to integrin downregulation on the cell surface. This mechanism is reminiscent of the relationship between gp 160, a human immunodeficiency virus glycoprotein, and its CD4 receptor (2, 4–6, 8, 9). The gp 160 reduces cell surface delivery of the CD4 molecule by interacting with it to form gp160–CD4 complexes in the endoplasmic reticulum that are thought to block the intracellular transport and delivery of CD4 to the plasma membrane (5, 9). Nonanchored gp160 does not produce this effect (8). Thus, these similarities suggest that the cytopathic effects observed in Ebola GP-expressing cells might be caused by such “receptor interference.”

To examine the potential role of  $\beta$ 1 integrins in Ebola virus entry, we tested 10 anti- $\beta$ 1 monoclonal antibodies for their ability to block the infectivity of vesicular stoma-

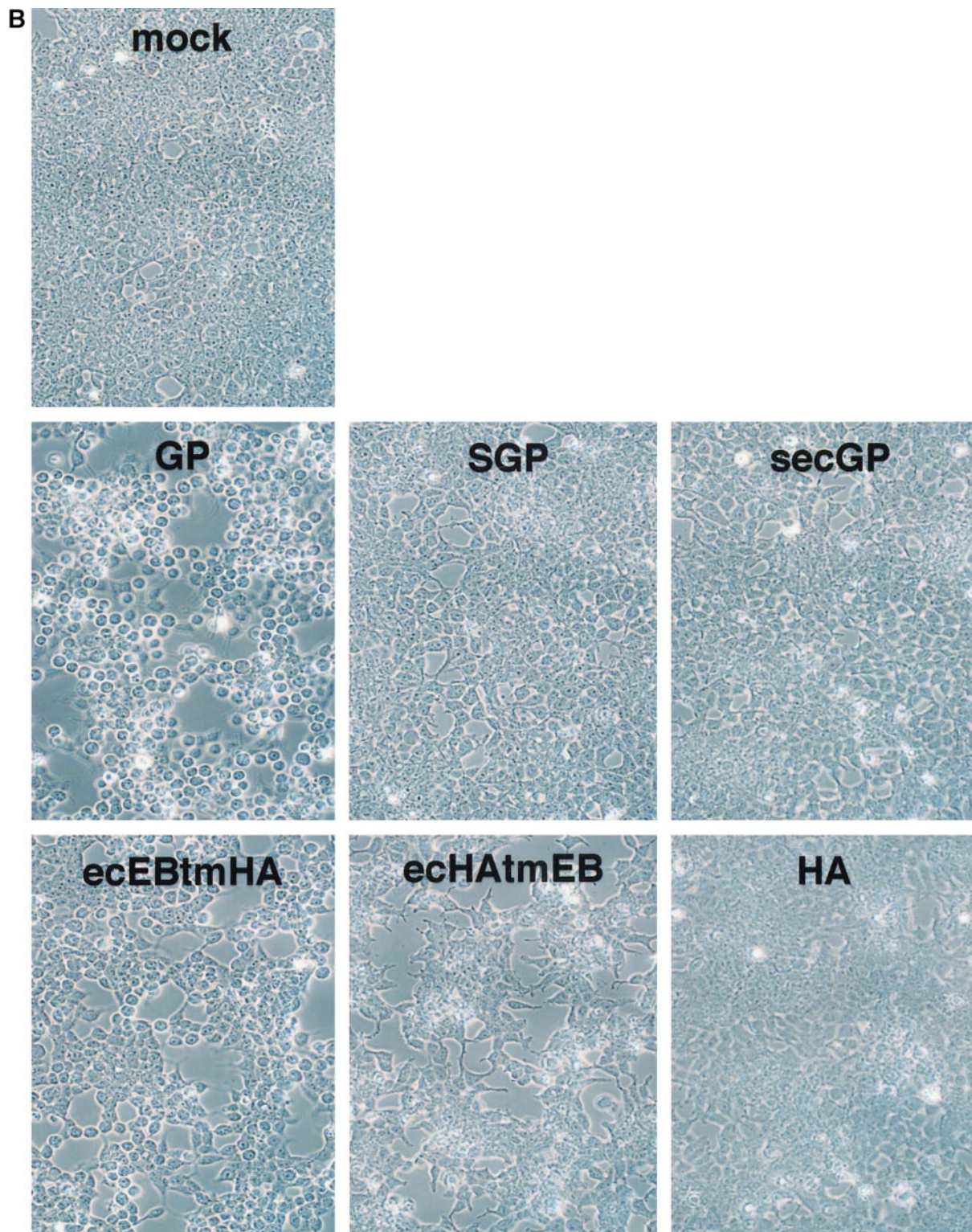


FIG. 1—Continued



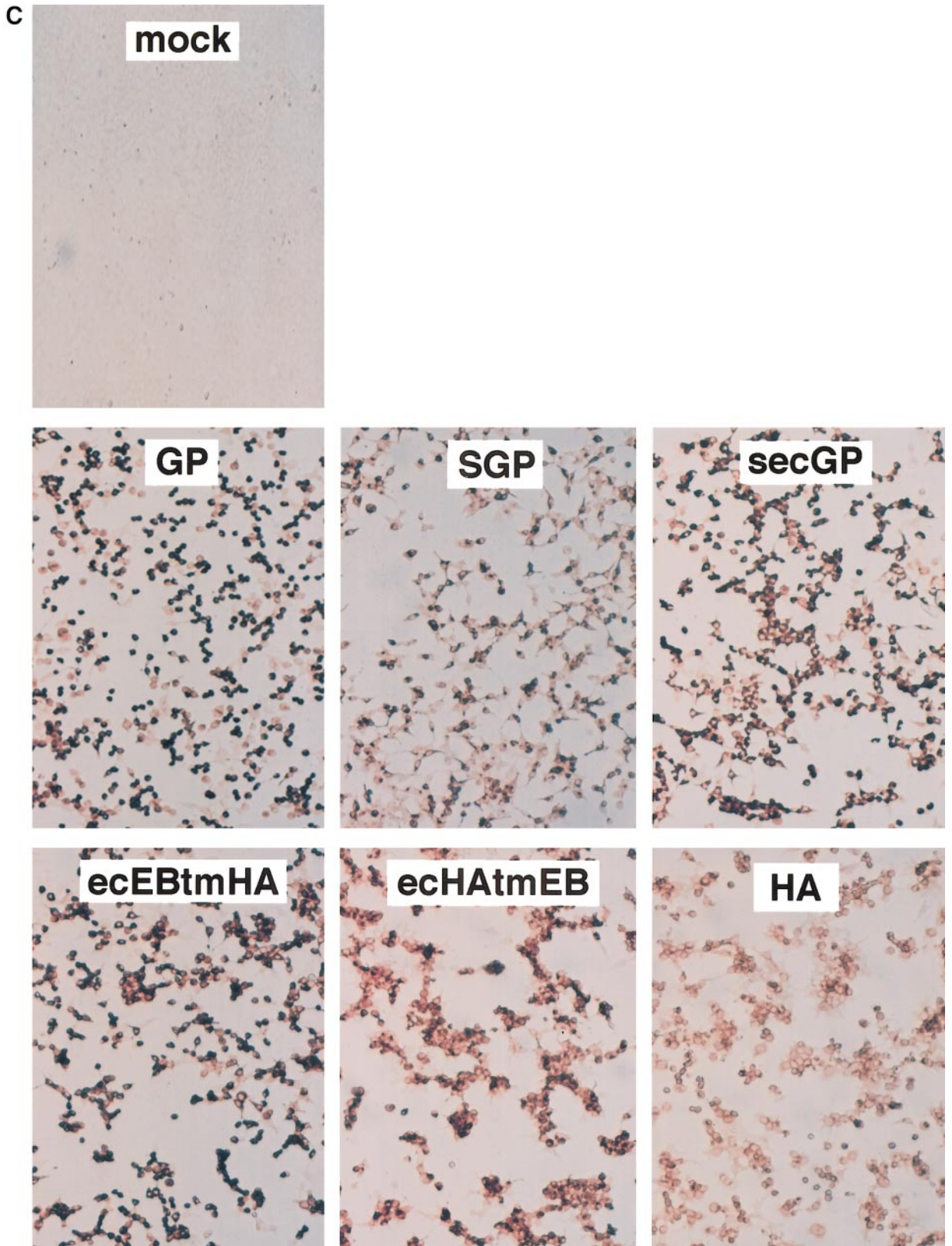
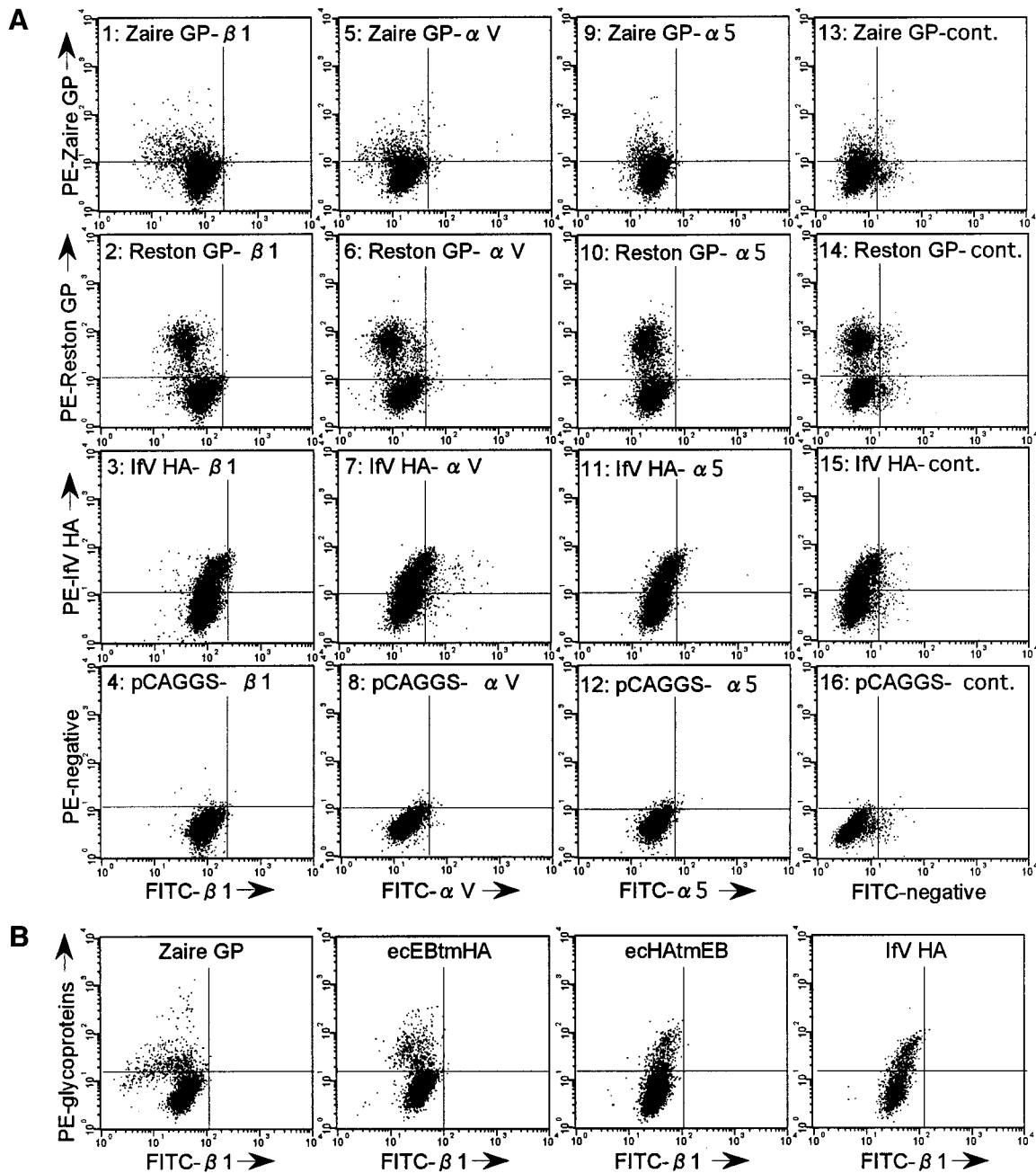


FIG. 1—Continued



**FIG. 2.** Downregulation of integrins by expression of Ebola GPs. 293T cells transfected with each plasmid were subjected to FACS analysis. Cells were incubated with rabbit polyclonal antibodies to Zaire GP/SGP or influenza virus, A/turkey/Ontario/7732/66 (H5N9), and with respective antihuman integrin mouse monoclonal antibodies (Chemicon), followed by incubation with secondary antibodies conjugated with phycoerythrin (PE) and FITC, respectively.

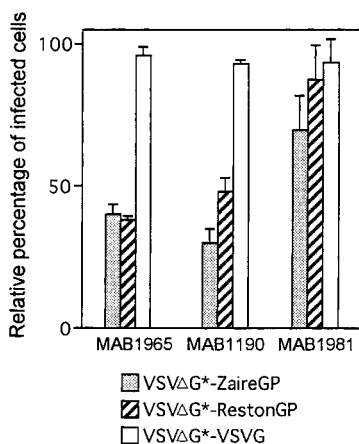
titis virus (VSV) pseudotyped with Ebola GP. Two of the antibodies (MAB1965 and 1190) markedly blocked infection by VSV $\Delta$ G\*-ZaireGP and VSV $\Delta$ G\*-RestonGP, while others (e.g., MAB1981) did not (Fig. 3). VSV $\Delta$ G\*-VSVG infectivity was not appreciably reduced by any of the antibodies. These data suggest that cell surface integrins, especially those in the  $\beta$ 1 group, are likely participants in Ebola virus entry into host cells. This hypothesis was examined further by testing the inhibitory effects

of purified integrins on the infectivity of the pseudotyped VSVs. As shown in Fig. 4, of three commercially available heterodimer integrins ( $\alpha$ 5 $\beta$ 1,  $\alpha$ v $\beta$ 3, and  $\alpha$ v $\beta$ 5; Triton X-100 or octyl- $\beta$ -D-glucopyranoside formulation, Chemicon), only  $\alpha$ 5 $\beta$ 1 inhibited the infectivity of VSV $\Delta$ G\*-ZaireGP and VSV $\Delta$ G\*-RestonGP; none altered VSV $\Delta$ G\*-VSVG infectivity, although  $\alpha$ v $\beta$ 3 did produce slight inhibition of the VSV $\Delta$ G\*-ZaireGP and VSV $\Delta$ G\*-RestonGP infection. These results, together with the antiviral activ-

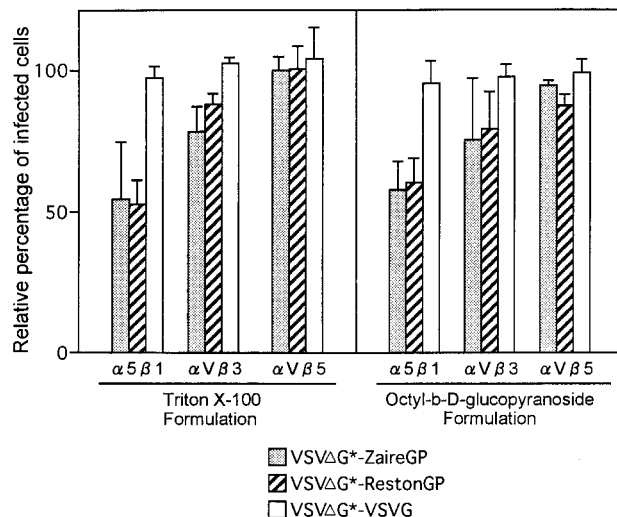
ity of the anti-integrin antibodies, support a contribution from the  $\beta 1$  integrins to cellular entry of Ebola viruses.

One putative receptor for the filoviruses is the asialoglycoprotein receptor found exclusively on hepatocytes (1). However, since filoviruses are pantropic and cell lines lacking this receptor are susceptible to the virus (13, 14), other receptors must exist. Our previous study showed that a cell surface glycoprotein with N-linked oligosaccharide side chains plays an important role in virus entry, presumably serving as a receptor or a cofactor (14). Taken together with our current data, we hypothesize that integrins are involved in Ebola virus entry.

The picture emerging from this study is that the integrins, the  $\beta 1$  molecules especially, may intracellularly interact with GPs during their movement through the intracellular membrane traffic, resulting in downregulation of these receptors and eventually cell rounding and detachment. Since integrins also participate in cell-cell interactions involving lymphocytes, their reduced expression in cells infected with Ebola virus could affect the efficiency of T-cell binding to the infected cells (3), which may hinder the induction of adequate immune responses to the virus. Further elucidation of how the Ebola GP downregulates integrin molecules should provide important additional insight into the mechanism of Ebola virus entry.



**FIG. 3.** Inhibition of pseudotyped VSV infection by anti- $\beta 1$  integrin antibodies. 293 cells were preincubated with anti- $\beta 1$  integrin monoclonal antibodies, MAB1965 (Chemicon), MAB1981 (Chemicon), and MAB1190 (Serotec) (ascites, 1:50 dilution), for 2 h at 37°C, and then infected with VSV pseudotyped with Zaire GP (VSV $\Delta$ G\*-ZaireGP), Reston GP (VSV $\Delta$ G\*-RestonGP), or VSV G (VSV $\Delta$ G\*-VSVG), as described previously (14). Briefly, appropriately diluted viruses ( $10^5$  infectious units/ml) were inoculated into cell monolayers, followed by a 1-h incubation. Infectivity was then determined by counting the cells expressing green fluorescent protein, which was encoded by the VSV $\Delta$ G genome. The number of infected cells treated with normal rabbit or mouse serum was set at 100. Each bar represents the mean percentage of infected cells  $\pm$  standard deviation in three independent experiments.



**FIG. 4.** Inhibitory effects of integrins on the infectivity of VSV pseudotyped with the Ebola GP. Triton X-100 and Octyl- $\beta$ -D-glucopyranoside present in the commercial integrin preparations (Chemicon) were removed with Bio-Beads SM-2 (Bio-Rad) or Centricon (Amicon). Each virus was mixed with purified integrins (100  $\mu$ g/ml in PBS), followed by a 1-h incubation at room temperature, and then inoculated into 293 cell monolayers. Under the experimental conditions used, we did not observe any effect on cells upon adding the integrin preparations. Each bar represents the mean percentage of infected cells  $\pm$  standard deviation in three independent experiments.

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