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Role of the Cytoplasmic Domains of Viral Glycoproteins in Antibody-Induced Cell Surface Mobility

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We have investigated the role of the cytoplasmic domains of the influenza virus hemagglutinin (HA) and the parainfluenza virus type 3 (PI3) fusion (F) glycoproteins as a determinant of their ability to undergo antibody-induced redistribution on plasma membranes. The viral envelope genes were truncated in their cytoplasmic domains by using oligonucleotide-directed mutagenesis and expressed by using recombinant vaccinia viruses. In HeLa cells, the truncated HA (HAt), like the full-length HA, did not cap in response to specific antibody. In CV-1 cells, HAt showed patchy surface immunofluorescence with few caps, whereas full-length HA exhibited capping in many cells in response to bivalent antibody. Quantitation of cap formation indicated a sevenfold decrease in the frequency of capping of HAt in comparison with full-length HA. Similarly, truncated F also exhibited a significant decrease in cap formation in comparison with full-length F. These results indicate that the ability of influenza virus HA and PI3 F to undergo redistribution in response to bivalent antibody has been altered by truncation of the viral glycoproteins and suggest that capping may involve interactions between the cytoplasmic domain of the viral glycoproteins and host cell components.

Many viral glycoproteins are mobile on surfaces of infected cells and may be redistributed into patches and caps in response to treatment with polyvalent ligands. We previously reported that there are significant viral and host cell-dependent differences in the ability of viral glycoproteins to undergo lateral redistribution in response to specific bivalent antibody. Influenza virus hemagglutinin (HA) and the G protein of vesicular stomatitis virus were readily found to cap when expressed on the surfaces of CV-1 cells, whereas neither glycoprotein was redistributed upon treatment of HeLa cells with specific bivalent antibody (15). Because these cell-dependent differences in capping might be due to a structural property of the glycoprotein itself or result from differences in interactions between the viral envelope proteins with other viral internal proteins in various host cells, we expressed viral glycoproteins in the absence of other viral components by using recombinant vaccinia viruses. The vaccinia virus-expressed proteins were found to exhibit behavior similar to that of glycoproteins in HeLa and CV-1 cells infected with wild-type viruses (15). These results indicated that the observed differences in capping were an intrinsic property of the viral glycoprotein molecules and not the result of interaction with other viral components.

Because the cytoplasmic domain of the proteins would be a likely site for interaction with cellular components that might modulate capping, we have investigated the role of the cytoplasmic domains in antibody-induced redistribution. The influenza virus HA and parainfluenza virus type 3 (PI3) fusion (F) proteins were compared because HA exhibits cell-dependent differences in capping whereas PI3 F was observed to cap in all cell types tested. In addition, we considered that the inability of influenza virus HA to cap in HeLa cells might be caused by the cytoplasmic tail of the glycoprotein being anchored to cell membrane or cytoskeletal proteins (7, 25) or alternatively by the inability of HA to interact with host cell components via its cytoplasmic domain.

HAt and Ft are expressed on cell surfaces. Cloned HA and F genes were truncated by using oligonucleotide-directed mutagenesis via polymerase chain reaction, and the nucleotide sequences of the genes were confirmed by limited sequence analysis using dideoxynucleotide chain termination (17). Intact and truncated influenza virus HA and PI3 F proteins were initially expressed from recombinant plasmids in HeLa T4 cells by using the vaccinia virus T7 expression system (9), and the viral polypeptides were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). Full-length HA with a molecular mass of 77 kDa and truncated HA (HAt) were expressed at similar levels. A difference in electrophoretic mobility of HA and HAt was not detected, probably because the molecular mass difference of 1.1 kDa is below the limit of resolution. Full-length F and truncated F (Ft) proteins were cleaved in HeLa T4 cells, and the F1 subunit of Ft migrated faster, corresponding to the predicted molecular mass difference from the full-length F1 subunit of 2.8 kDa.

Recombinant vaccinia viruses expressing influenza virus HAt (VVHAt) and PI3 Ft (VVFt) were constructed by previously described procedures (2, 23), and surface expression of the truncated glycoproteins was observed on infected cells by indirect immunofluorescence microscopy as previously described (15). Levels of expression of the full-length and truncated HA and F glycoproteins by recombinant vaccinia viruses on cell surfaces were compared by fluorescence-activated cell sorting (FACS) analysis. In CV-1 cells, HA exhibited a mean fluorescence intensity profile of 680 (Fig. 1a, right), compared with 629 for HAt (Fig. 1b, right). The similarity of the FACS profiles of HA and HAt (Fig. 1c) indicated that the two glycoproteins were expressed at similar levels on cell surfaces. These results are similar to those in previous reports in which it was concluded that the cytoplasmic domain of influenza virus HA was not required for intracellular transport (4, 5). The fluorescence intensity profile of full-length F was 402 (Fig. 1d, right), while that of

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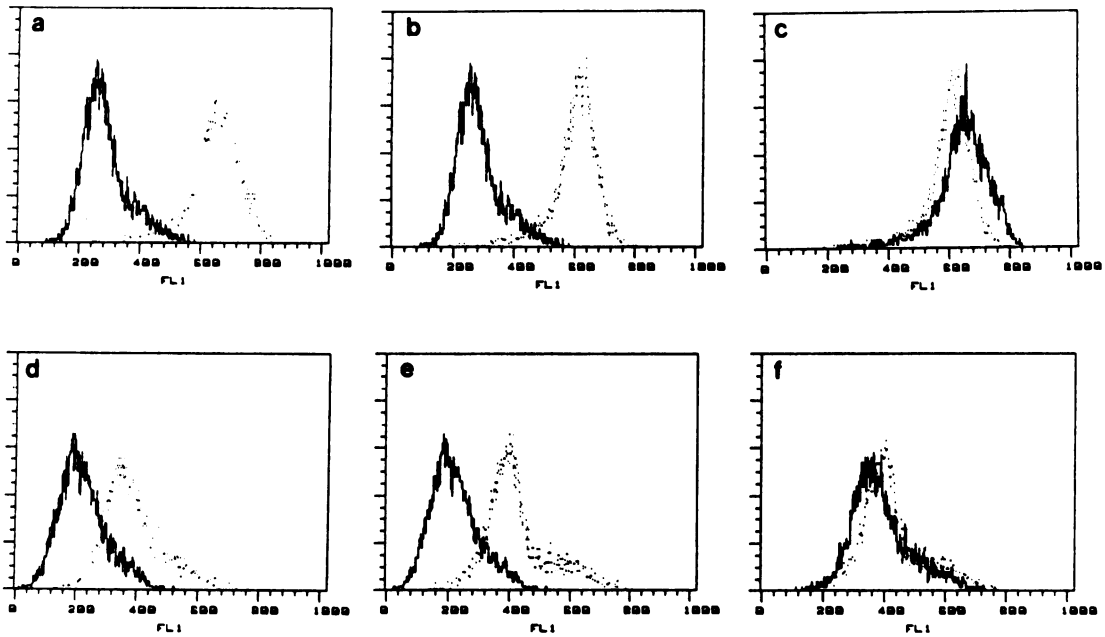

FIG. 1. FACS profiles comparing surface expression of full-length and truncated viral glycoproteins on HeLa and CV-1 cells. Cells were infected with recombinant vaccinia viruses expressing either vaccinia virus vector proteins (control) or full-length or truncated HA or F. Cells were immunolabeled as described previously (15) with anti-HA or guinea pig anti-PI3 antibodies and anti-Ig-FITC and then fixed with 1% paraformaldehyde in PBS. Cells were analyzed at a density of 10^6 cells per ml. Fluorescence intensity is given on the horizontal scale. Each panel compares two fluorescence profiles (left to right) which are identified as follows: (a) vector and full-length HA, (b) vector and HAt, and (c) HA (dots) and HAt (line) in CV-1 cells; (d) vector and full-length F, (e) vector and Ft, and (f) full-length F (line) and Ft (dots) in HeLa cells.

Ft was 433 (Fig. 1e, right), which also indicated similar levels of surface expression (Fig. 1f). Kolmogorov-Smirnov two-sample statistical analysis (24) indicated that the FACS profiles of F and Ft were distinct from the vaccinia virus vector profile (Fig. 1d and e, left) at the 99% confidence level. These results indicated that full-length HA was expressed at a slightly higher level than HAt on cell surfaces, whereas Ft was expressed at a slightly higher level than full-length F on HeLa cells. These results thus indicate that the 22 C-terminal amino acids of the cytoplasmic tail of the F protein are not required for intracellular transport and expression of the truncated protein on the plasma membrane.

Antibody-induced redistribution of truncated viral glycoproteins. To investigate the role of the cytoplasmic domain of the viral glycoproteins on the ability of these proteins to undergo antibody-induced redistribution on the surfaces of infected cells, we compared the surface fluorescence patterns of the glycoproteins after incubation with bivalent antibody at 37°C as previously described (15). Briefly, monolayer cells were infected with recombinant vaccinia virus at a multiplicity of infection of 10, suspended at 2 h postinfection in nonenzymatic dissociation buffer (Sigma), and maintained in Joklik's medium plus 2% calf serum at 37°C on a Labquake shaker. Capping experiments were initiated at 6 h postinfection, when cells were found to be expressing high levels of viral proteins on their surfaces as determined by immunofluorescence. Control experiments previously showed that the vaccinia virus infection did not affect the ability of viral glycoproteins to cap in response to specific antibody (15). Cells expressing HA or HAt were incubated with monoclonal anti-HA antibody for 30 min on ice, rinsed with phosphate-buffered saline (PBS), and incubated with anti-fluorescein isothiocyanate (FITC)-conjugated immunoglobulin (Ig) antibody for 30 min either on ice (control cells) or at 37°C. Cells were maintained at 4°C during all subsequent rinse and pelleting steps and immediately fixed with 1% paraformaldehyde in PBS at the conclusion of the experiment. Cells expressing F or Ft were incubated with either monoclonal anti-F antibody or anti-PI3 antibody on ice for 30 min, rinsed, and incubated with anti-Ig-FITC for 60 min either on ice or at 37°C and fixed as described above. VVHAt was not found to cap in HeLa cells after incubation with antibody at 37°C (Fig. 2d). This lack of antibody-induced redistribution was similar to the result we previously reported for full-length HA in HeLa cells (15) (Fig. 2b). Thus, truncation of the cytoplasmic domain of influenza virus HA did not alter the restriction in capping observed in HeLa cells.

Under similar experimental conditions in CV-1 cells, recombinant HAt predominantly exhibited a patchy pattern of surface immunofluorescence on the cell surfaces at 37°C, indicating that the lateral redistribution of HAt had been altered (Fig. 3d). In contrast, under the same experimental conditions, full-length HA was found to redistribute into cap structures on the majority of cells (Fig. 3b). In Fig. 3d, the patches formed by HAt are very large. This observation suggests that the lateral movement of HAt is arrested at the stage of patch formation and that patches of viral antigen-antibody complexes are unable to condense into a cap structure. Patching is considered to be different from capping in that it does not require metabolic energy and is not affected by cytoskeleton-disrupting drugs (14, 18). The differences in antibody-induced redistribution between intact and truncated HA glycoproteins were quantitated by counting the number of capped cells as a percentage of the total

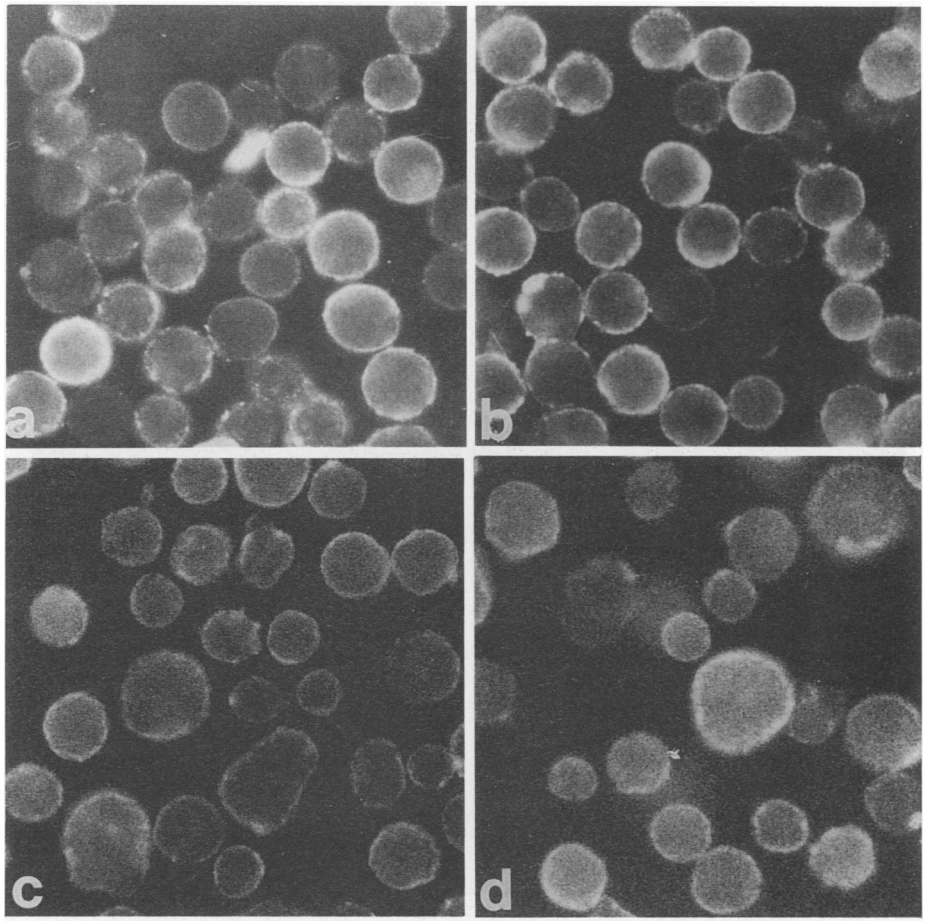

FIG. 2. Lack of effect of truncation of HA on antibody-induced redistribution in HeLa cells. VVHA (a) and VVHAt (c) show diffuse surface fluorescence at 6 h postinfection after immunolabeling on ice. Similar diffuse fluorescence is seen with VVHA (b) and VVHAt (d) after immunolabeling at 37°C. The differences in fluorescence intensity in micrographs of control cells are due to differences in photographic exposure times and printing conditions.

number of fluorescent cells (11, 12). At 37°C, 72% of the VVHA-infected CV-1 cells were found to exhibit well-defined cap structures, whereas only 10% of the VVHAt-infected cells were found to undergo capping (Table 1). Incubation of VVHAt-infected CV-1 cells for 60 min instead of the usual 30 min in the presence of antibody at 37°C did not significantly alter the percentage of capped cells (Table 1). These differences in the ability of HA and HAt to redistribute in response to specific antibody indicate that the cytoplasmic domain of influenza virus HA is necessary for the protein's ability to undergo antibody-directed redistribution on CV-1 cell surfaces.

The distribution of the intact and truncated PI3 F proteins on HeLa cells after incubation in the presence of antibody is shown in Fig. 4. Control cells which were infected with wild-type PI3, VVF, or VVFt exhibited diffuse surface fluorescence when immunostained for F and incubated on ice (Fig. 4a to c). At 37°C, both PI3- and vaccinia virus-expressed F exhibited patching and capping in HeLa cells (Fig. 4d and e), whereas Ft showed predominantly diffuse surface immunofluorescence, indicating its inability to cap (Fig. 4f). Quantitation of cap formation by F and Ft proteins in HeLa cells at 37°C also demonstrated a significant reduction in cap formation by the Ft proteins (Table 1). These results indicate that, as for influenza virus HA, the cytoplasmic domain of the glycoprotein plays a role in the antibody-induced redistribution of the PI3 F protein.

Membrane proteins may undergo random motion which is involved in lateral diffusion or directed motions such as capping or be laterally constrained (6). Fluorescence recovery after photobleaching (FRAP) has been used to measure the passive, uninduced lateral diffusion of membrane proteins, whereas microscopic observation has been used to investigate the active, energy-dependent capping of proteins in response to specific cross-linking agents such as bivalent antibody or ligands (16). Capping is thought to involve cell cytoskeletal components (3, 10), and patching is considered to be different from capping in that it does not require metabolic energy and is not affected by cytoskeleton-disrupting agents (14, 18). Several investigators have reported that truncation of the cytoplasmic domains of vesicular stomatitis virus G (19), major histocompatibility complex class I antigens (8), and epidermal growth factor receptor (13) did not alter their lateral diffusion as measured by FRAP. In contrast to these results, the cytoplasmic domain was reported to play a role in the lateral diffusion observed with major histocompatibility complex class II antigens (22). There are few published reports on the role of protein

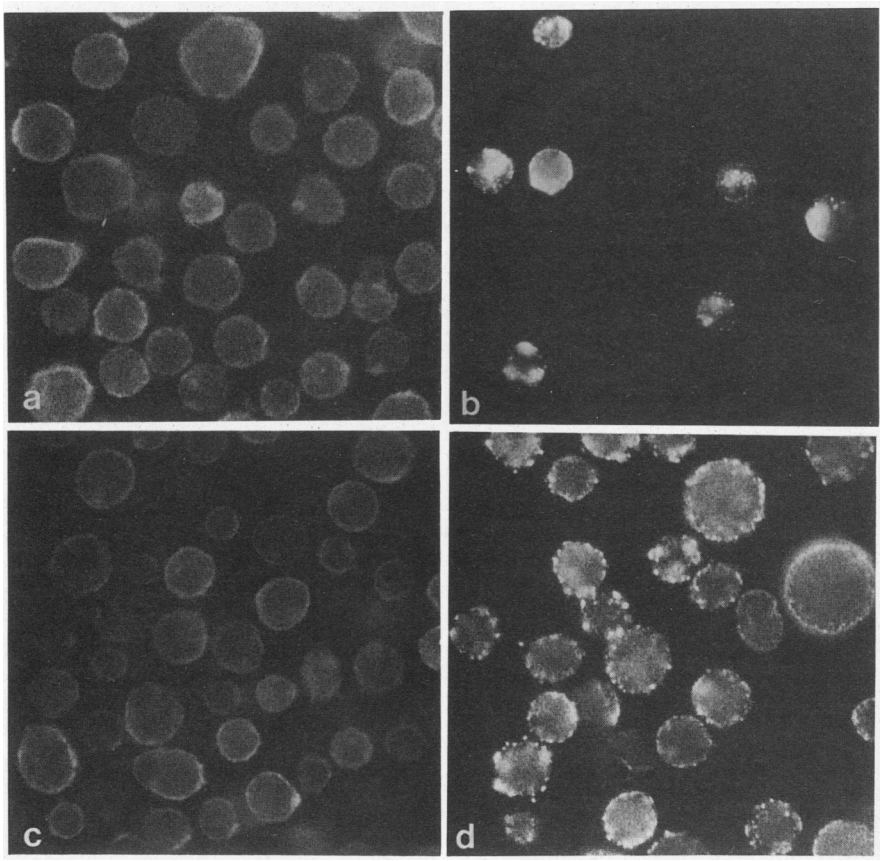

FIG. 3. Effect of truncation on the capping of VVHA in CV-1 cells. VVHA (a) and VVHAt (c) show diffuse surface fluorescence after immunostaining and incubation on ice. Capping of VVHA (b) and patching with some capping of VVHAt (d) as seen in CV-1 cells at 37°C.

structure in ligand- or antibody-induced movement of transmembrane glycoproteins on cell surfaces, and correlations between lateral mobility as measured by FRAP and ligand-induced redistribution cannot be made with certainty.

Edidin et al. (7) reported that gold bead-labeled major histocompatibility complex class I antigens encountered dynamic barriers to lateral movement on the cell surface which were located primarily on the cytoplasmic face of the membrane in nonerythroid cells. These barriers are thought to be analogous to spectrin-containing structures which may partition the erythrocyte membrane into corrals (20). It was proposed that protein molecules diffuse freely within this metabolically active cytoskeletal meshwork but cannot cross the boundaries of the corral, thus exhibiting net constrained lateral diffusion (7). This model of protein mobility restriction is consistent with the process of protein patching on cell surfaces. Proteins within a microdomain on the plasma membrane may aggregate in the presence of cross-linking agents and form patches which would be constrained by the boundary of the corral without interaction with other underlying cytoskeletal structures. It has been suggested that once a minimum threshold patch size (1) or minimum thermodynamic energy barrier (21) is exceeded, the patched proteins could activate membrane-associated enzymes or generate a transmembrane signal which would trigger the direct or indirect binding of the patched proteins to cytoskeletal elements of the cell (1). The protein patch would move beyond the boundaries of the membrane microdomain in an energy- and temperature-dependent contractile process such as the sliding filament mechanism proposed by Bourguignon and Bourguignon (1), and protein capping would result. This model provides an attractive explanation for the observed differences in the capping of influenza virus HA in HeLa and CV-1 cells. Possibly the size of microdomains on HeLa cell surfaces prevents cross-linked HA patches from reaching the threshold size or energy limit and thus prevents triggering a linkage with cytoskeletal components or other membrane components associated with the cytoskeleton. An

TABLE 1. Effect of cytoplasmic tail truncation on the antibody-induced redistribution of viral glycoproteins[a]

Cell line	Time (min)	Fraction of capped cells (%)[b]			
		VVHA	VVHAt	VVF	VVFt
CV-1	30	396/551 (72)	53/514 (10)	ND[c]	ND
CV-1	60	384/529 (73)	54/491 (11)	ND	ND
HeLa	60	0/496 (0)	0/521 (0)	60/471 (12)	8/561 (1)

[a] See Materials and Methods for conditions used to cap cells.

[b] Data represent the number of cells with well-defined caps as a percentage of the total number of fluorescent cells counted which were expressing the surface viral antigen. Proteins which did not undergo capping exhibited diffuse fluorescence on the cell surface. In contrast, viral glycoproteins were considered to be capped when fluorescence was concentrated into 50% or less of the cell surface. Cells exhibiting patched antigens were not counted as capped cells.

[c] ND, not done.

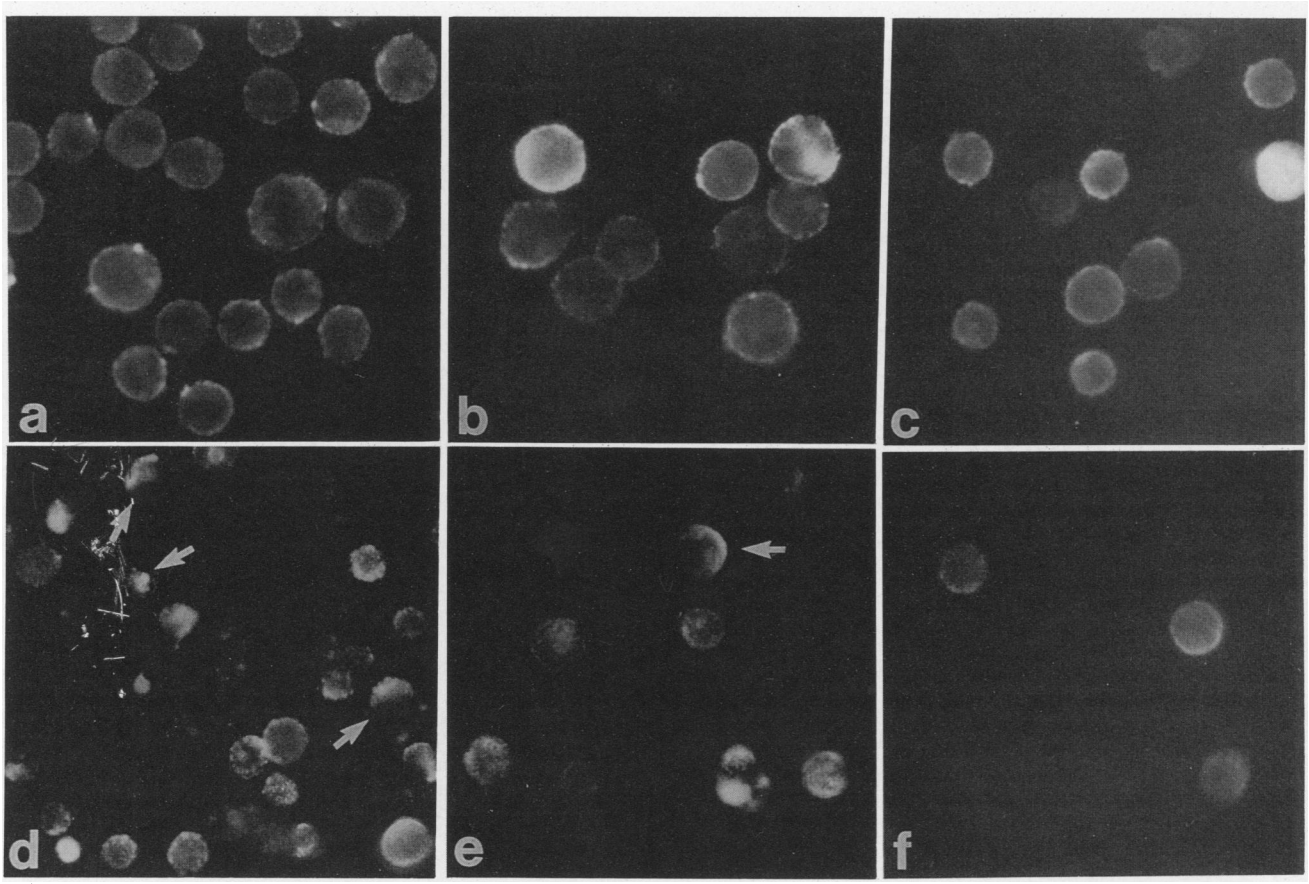

FIG. 4. Effect of truncation on the capping of the F glycoprotein. (a to c) Surface fluorescence of PI3-F (a), VVF (b), and VVFt (c) after immunolabeling on ice; (d to f) surface fluorescence of PI3 F (d), VVF (e), and VVFt (f) after incubation with antibody at 37°C. Arrows in panels d and e indicate capped cells.

alternative explanation is that even if cross-linked HA molecules form patches of the critical size, the HA may be unable to interact with host cell components underlying the HeLa cell surface. The net result would be the observed restriction of mobility of both full-length and truncated HA in HeLa cells. In such a system, truncation of the viral protein would not be expected to alter the inability of full-length HA to cap in HeLa cells, whereas it would be expected to have an effect on the capping of HA in CV-1 cells. This model would also be consistent with the observed antibody-induced redistribution of the PI3 fusion protein in both cell types, implying that the restriction of influenza virus HA mobility in HeLa cells is caused by an inability of HA to interact with cellular elements and not due to inability to reach a critical patch size based on the area of the microdomain. Although less likely, the possibility also exists that carboxy-terminal modifications of the HA and F glycoproteins might alter the conformation of the external domains of the proteins such that epitopes important for antibody to cross-link oligomeric glycoprotein structures would be altered. We are currently investigating interactions between the viral glycoprotein and host cell components to determine whether such interactions may be related to observed differences in protein capping.

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