JOURNAL OF VIROLOGY, Feb. 2004, p. 1552–1563 Vol. 78, No. 3
0022-538X/04/$08.00+0 DOI: 10.1128/JVI.78.3.1552–1563.2004

Cell-Type-Dependent Targeting of Human Immunodeficiency Virus Type 1 Assembly to the Plasma Membrane and the Multivesicular Body

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Received 1 August 2003/Accepted 7 October 2003

The human immunodeficiency virus type 1 (HIV-1) assembly-and-release pathway begins with the targeting of the Gag precursor to the site of virus assembly. The molecular mechanism by which Gag is targeted to the appropriate subcellular location remains poorly understood. Based on the analysis of mutant Gag proteins, we and others have previously demonstrated that a highly basic patch in the matrix (MA) domain of Gag is a major determinant of Gag transport to the plasma membrane. In this study, we determined that in HeLa and T cells, the MA mutant Gag proteins that are defective in plasma membrane targeting form virus particles in a CD63-positive compartment, defined as the late endosome or multivesicular body (MVB). Interestingly, we find that in primary human macrophages, both wild-type (WT) and MA mutant Gag proteins are targeted specifically to the MVB. Despite the fact that particle assembly in macrophages occurs at an intracellular site rather than at the plasma membrane, we observe that WT Gag expressed in this cell type is released as extracellular virions with high efficiency. These results demonstrate that Gag targeting to and assembly in the MVB are physiologically important steps in HIV-1 virus particle production in macrophages and that particle release in this cell type may follow an exosomal pathway. To determine whether Gag targeting to the MVB is the result of an interaction between the late domain in p6^{Gag} and the MVB sorting machinery (e.g., TSG101), we examined the targeting and assembly of Gag mutants lacking p6. Significantly, the MVB localization of Gag was still observed in the absence of p6, suggesting that an interaction between Gag and TSG101 is not required for Gag targeting to the MVB. These data are consistent with a model for Gag targeting that postulates two different cellular binding partners for Gag, one on the plasma membrane and the other in the MVB.

Retrovirus particle production is promoted by the viral structural protein Gag and, for viruses that follow the C-type assembly pathway, generally consists of four major steps: targeting of the Gag polyprotein precursor to the plasma membrane, Gag membrane binding, Gag multimerization, and finally the budding and pinching-off of the nascent virus particle from the cell surface (11, 23, 55). Concomitant with or immediately after the release of the nascent particle, processing of the Gag precursor proteins by the viral protease (PR) triggers a major change in virion morphology, a process known as maturation (11, 55). In the case of human immunodeficiency virus type 1 (HIV-1), the Gag precursor, Pr55^{Gag}, is cleaved by PR into four mature Gag proteins, i.e., p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), and p6, and two spacer peptides, i.e., p2 and p1 (11, 55). Functional domains that promote binding of Gag to membrane (membrane binding [M] domain), Gag multimerization (interaction [I] domain), and pinching-off of virus particles (late [L] domain) have been mapped in Pr55^{Gag} to the N-terminal portion of MA, the region spanning the C terminus of CA to the N terminus of NC, and p6, respectively (11, 55).

The molecular mechanisms by which the M and I domains mediate Gag membrane binding and multimerization have been largely determined (11, 55). L domain function has recently been the focus of intense investigation, and it is now well established that the L domain of p6 binds TSG101 (12, 45), a host protein involved in endosomal sorting and the delivery of a number of cellular proteins to the multivesicular body (MVB) (2). Details of how the p6-TSG101 interaction promotes the budding-off of virions from the cell surface are currently being elucidated in this and other laboratories. In contrast, the process by which Gag is targeted to the site of virus assembly remains largely uncharacterized. Studies conducted thus far have revealed that the viral determinant(s) that directs Gag to the plasma membrane is located in the MA domain. Deletion of large portions of HIV-1 MA causes accumulation of virus particles in the endoplasmic reticulum (ER) (9, 18) or promiscuous targeting of virus assembly to a variety of cellular membranes (33, 48, 60). MA mutations in other retroviruses, e.g., murine leukemia virus and Rous sarcoma virus, have also been shown to alter the destination to which Gag is targeted (24, 49, 52). Also in support of a role for MA in Gag targeting is the observation that the HIV-1 assembly defect in murine cells, which is accompanied by an apparent accumulation of Gag at intracellular sites, can be corrected by substituting murine leukemia virus MA for HIV-1 MA in HIV-1 Gag (4, 35, 47).

We and others (17, 25, 41, 62) have previously shown that amino acid substitutions in either the highly basic domain (residues 17 to 31) or residues 84 to 88 of HIV-1 MA redirect

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Gag to intracellular vesicles, leading to impaired production of extracellular virus particles. Importantly, particle formation and virion maturation still occur within these vesicles, which are positive for trans- and/or post-Golgi markers (17, 41). The isolation and characterization of viral revertants derived from a residue 86 mutant suggested that the highly basic domain and residues 84 to 88 are functionally or structurally linked (39, 41). Since the highly basic domain is apparently exposed on the surface of MA whereas residues 84 to 88 are buried within the globular domain of the protein (26), it appears likely that the highly basic domain functions as the primary signal for Gag targeting to the plasma membrane.

The targeting phenotype of MA mutants has been analyzed mainly in HeLa or COS cells, which, like T cells, support wild-type (WT) virus assembly predominantly on the plasma membrane (21, 22). However, it has long been appreciated that in primary macrophages HIV-1 particle assembly normally takes place in intracellular vesicles rather than on the plasma membrane (42). A recent immunoelectron microscopy (immuno-EM) study (46) observed that the intracellular organelle in which HIV-1 assembly occurs in macrophages is positive for CD63, a tetraspan protein localized largely to the MVB (8). Despite the fact that infection of macrophages plays a central role in HIV-1 replication, transmission, and pathogenesis in vivo (5, 20, 29, 31, 37), the targeting of HIV-1 assembly in this cell type remains poorly characterized. It is unclear, for example, whether virus assembly in the MVB represents a productive pathway for extracellular particle production in macrophages and which region(s) of $Pr55^{Gag}$ determines Gag targeting to this organelle.

To elucidate the molecular mechanisms underlying Gag targeting, in this study we sought to compare Gag behavior among different cell types. We first defined the site of intracellular assembly in HeLa and T cells of Gag mutants containing substitutions in the highly basic domain or residues 84 to 88 of MA. We observed that these mutant Gag proteins displayed a striking colocalization with the MVB marker CD63. In macrophages, not only WT Gag but also MA mutant Gag specifically colocalized with CD63. Despite the difference in their localization, WT Gag proteins were released extracellularly from macrophages and HeLa cells with similar efficiencies. Importantly, Gag localization to the CD63-positive compartment was not affected by the lack of p6 in either HeLa cells or macrophages, suggesting that MVB targeting is not driven by an interaction between p6 and the MVB sorting machinery (e.g., TSG101). We suggest that two distinct pathways for HIV-1 Gag targeting exist, one to the plasma membrane and the other to the MVB. Our data suggest that MVB targeting is the major physiological pathway for extracellular virus production in primary macrophages.

MATERIALS AND METHODS

Cells, transfections, and infections. HeLa and Jurkat cells were cultured as previously described (15). Monocyte-derived macrophages were prepared by culturing elutriated monocytes (14) in RPMI 1640 medium supplemented with 10% human AB type serum for 7 days. Transfection of HeLa cells by the calcium phosphate method was performed as previously described (15). Infection of HeLa cells and macrophages with virus stocks pseudotyped with the vesicular stomatitis virus G glycoprotein (VSV-G) was performed by culturing cells for 9 h with high-titer virus stocks at 30 cpm of reverse transcriptase activity/cell.

Plasmids and virus preparation. Molecular clones expressing Gag mutants, i.e., pNL4-3/85YG (17), pNL4-3/29KE/31KE (41), pNL4-3/85YG/p6$^-$ (41), and pNL4-3/L1term (27), were described previously. A molecular clone expressing the 29KE/31KE mutant Gag in the absence of Env, pNL4-3/29KE/31KE/KFS, was constructed by exchanging the *Eco*RI-*Bam*HI fragment of pNL4-3/29KE/31KE with the corresponding fragment from pNL4-3/KFS (13, 16). Construction of pCMVNLGagPolRRE by using pCMVGagPolRRE (a kind gift from D. Rekosh [53]) was described previously (38). Plasmids expressing a C-terminally Flag-tagged $Pr55^{Gag}$ and the 29KE/31KE MA mutant derivative were described previously (41). pHCMV-G (61) was generously provided by J. Burns (University of California, San Diego). VSV-G-pseudotyped virus stocks were prepared by transfecting HeLa cells with pCMVNLGagPolRRE-, pHCMV-G-, and pNL4-3-derived molecular clones.

Antibodies, fluorescent reagents, and immunostaining. The following antibodies were obtained from the indicated sources: mouse monoclonal antibody which recognizes p17 (MA) but not $Pr55^{Gag}$, Advanced Biotechnologies (Columbia, Md.); rabbit anticalreticulin antibody and rabbit anti-Flag antibody, Affinity Bioreagents (Golden, Colo.); mouse anti-GM130 antibody and mouse anti-EEA1 antibody, Transduction Laboratories (Lexington, Ky.); sheep anti-TGN46 antibody, Serotec (Oxford, United Kingdom); mouse anti-CD63 antibody and rabbit anti-Rab7 antibody, Santa Cruz Biotechnology (Santa Cruz, Calif.); mouse anti-lysobisphosphatidic acid (anti-LBPA) monoclonal antibody (30), a kind gift from J. Gruenberg (University of Geneva, Geneva, Switzerland); HIV immunoglobulin (Ig), the National Institutes of Health AIDS Research and Reference Reagent Program; and Texas red-conjugated anti-mouse IgG Fcγ, anti-rabbit IgG, and anti-sheep IgG antibodies, Jackson Immunoresearch Laboratories (West Glove, Pa.). Fluorescence-conjugated wheat germ agglutinin (WGA), Alexa 594-conjugated transferrin, LysoTracker Red, and Zenon One Alexa 488 and 594 mouse IgG1 labeling kits were obtained from Molecular Probes (Eugene, Oreg.).

Immunostaining of cells was performed as described previously (40, 41) with some modifications. Briefly, transfected or infected cells grown in chamber slides (Nunc) were rinsed once with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in 100 mM sodium phosphate buffer (pH 7.2) for 20 min. All procedures were carried out at room temperature unless otherwise noted. After being washed four times with PBS, cells were permeabilized with PBS containing 0.1% Triton X-100 for 2 min, followed by washing with PBS three times. Subsequently, cells were incubated with 0.1 M glycine in PBS for 10 min and blocked with 3% bovine serum albumin in PBS (BSA-PBS) for 30 min. The cells were then incubated with primary antibodies diluted appropriately in BSA-PBS for 1 h, washed with PBS three times, incubated with secondary antibodies appropriately diluted in BSA-PBS for 30 min, and washed with PBS three times. For localization studies using Alexa 594-conjugated transferrin or LysoTracker Red, cells were incubated with these reagents for 30 min at 37°C before fixation based on the manufacturer's recommendations. For double staining with two different mouse monoclonal antibodies, antibodies were fluorescently labeled with the Zenon One Alexa 488 or 594 IgG1 labeling kit according to the manufacturer's protocol and cells were incubated sequentially with each antibody. To avoid dissociation and rebinding of Zenon One reagents, after each incubation with the fluorescently labeled antibody, cells were incubated in the fixation buffer (above) for 10 min followed by washing with PBS three times. Alternatively, cells were stained with one of the monoclonal antibodies followed by incubation with Texas red-conjugated anti-mouse IgG Fcγ and blocked by normal mouse IgG (Santa Cruz). Subsequently, cells were stained with another monoclonal antibody labeled with Zenon One Alexa 488 as indicated above. After staining, cells were mounted with Fluoromount G (Electron Microscopy Sciences, Fort Washington, Pa.) and examined with a Leica TCS/NT SP1 laser scanning microscope.

Metabolic labeling and immunoprecipitation. One day after infection with pseudotyped viruses, cells were metabolically labeled with [^{35}S]methionine-cysteine in RPMI 1640 medium lacking methionine and cysteine and supplemented with 10% fetal bovine serum (for HeLa cells) or human AB type serum (for macrophages) for 16 h. Preparation of cell lysates, pelleting of virions in the ultracentrifuge, and immunoprecipitation of cell- and virion-associated proteins with HIV Ig have been detailed previously (17).

RESULTS

MA mutations retarget HIV-1 Gag to a CD63-positive organelle. We previously reported that whereas WT HIV-1 Gag is directed predominantly to the plasma membrane, amino acid substitutions in the highly basic domain (residues 17 to 31)

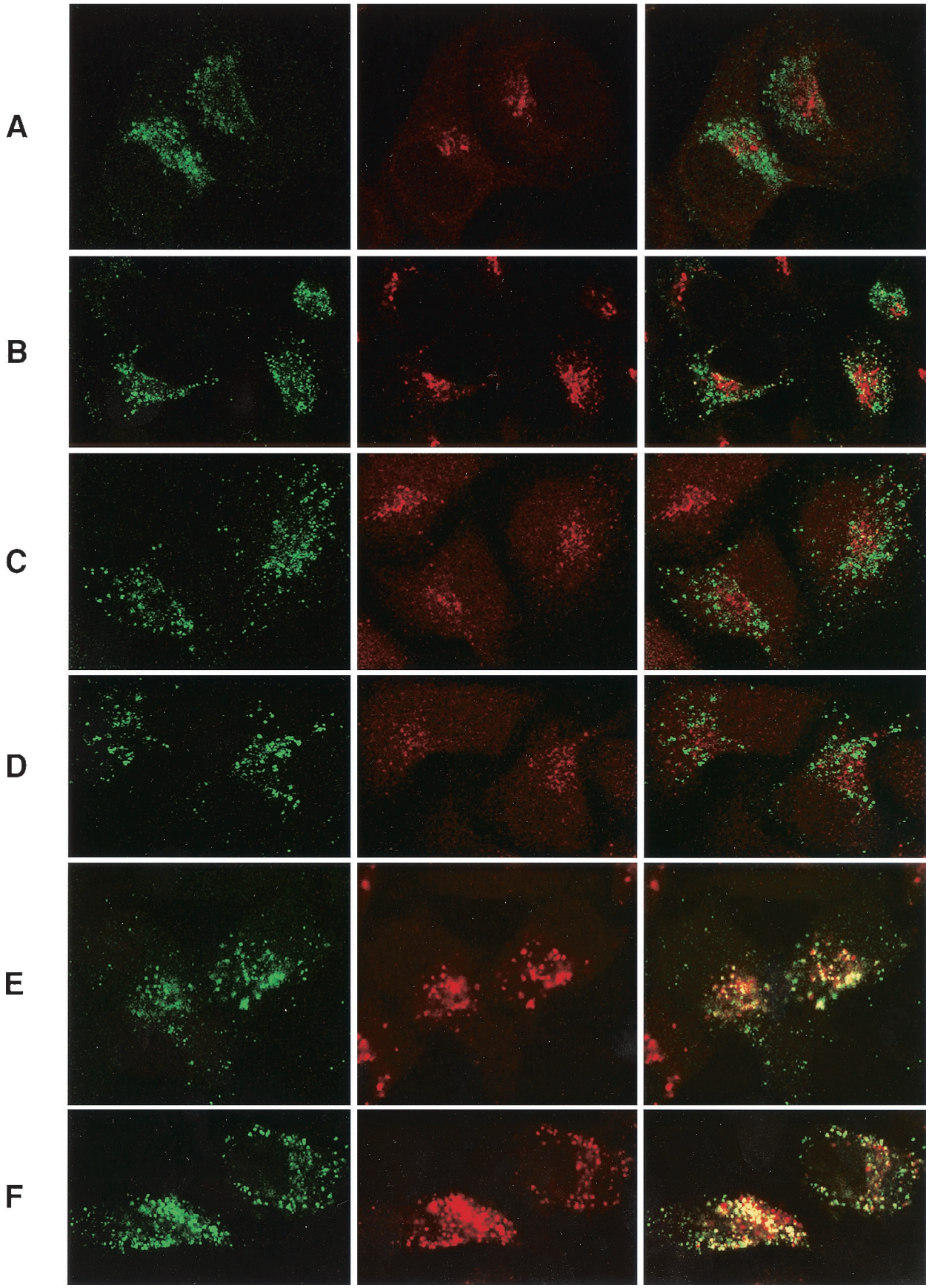
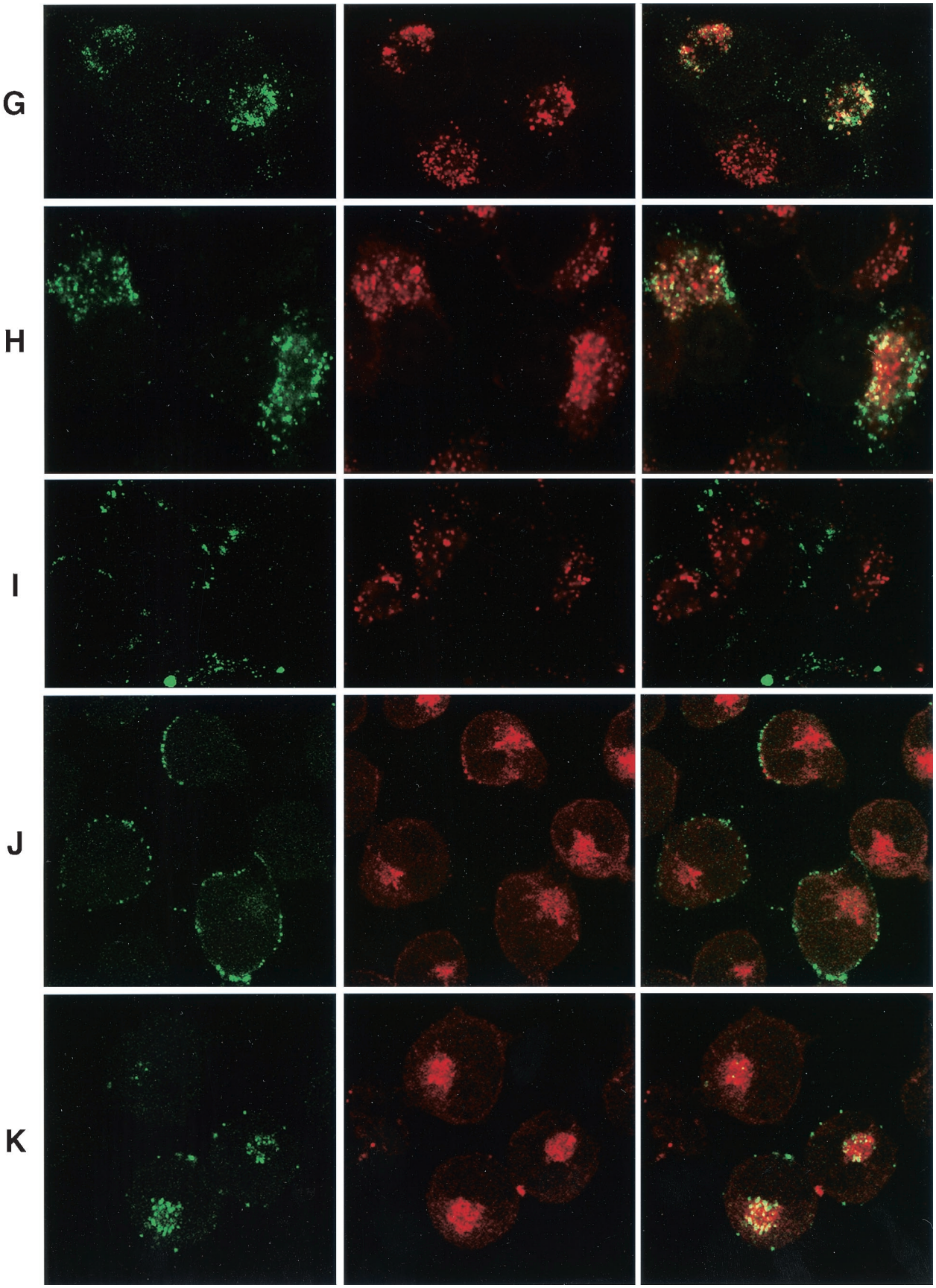


FIG. 1. Mutant Gag proteins colocalize with markers for the MVB in HeLa and Jurkat T cells. HeLa cells were transfected with pNL4-3/29KE/31KE (A to C, E, G, and H), pNL4-3/85YG (D and F), or WT pNL4-3 (I). Jurkat cells were infected with VSV-G-pseudotyped viruses expressing WT (J) or 29KE/31KE mutant (K) Gag proteins. Cells were stained with anti-MA antibody (left columns) and a variety of organellar



markers (middle columns) as detailed in Materials and Methods. The markers used were anti-GM130 (A), anti-TGN46 (B), anti-EEA1 (C and D), anti-CD63 (E, F, I, J, and K), anti-LBPA (G), and LysoTracker (H). Merged images of MA and organellar signals, with colocalization indicated in yellow, are shown in the right columns.

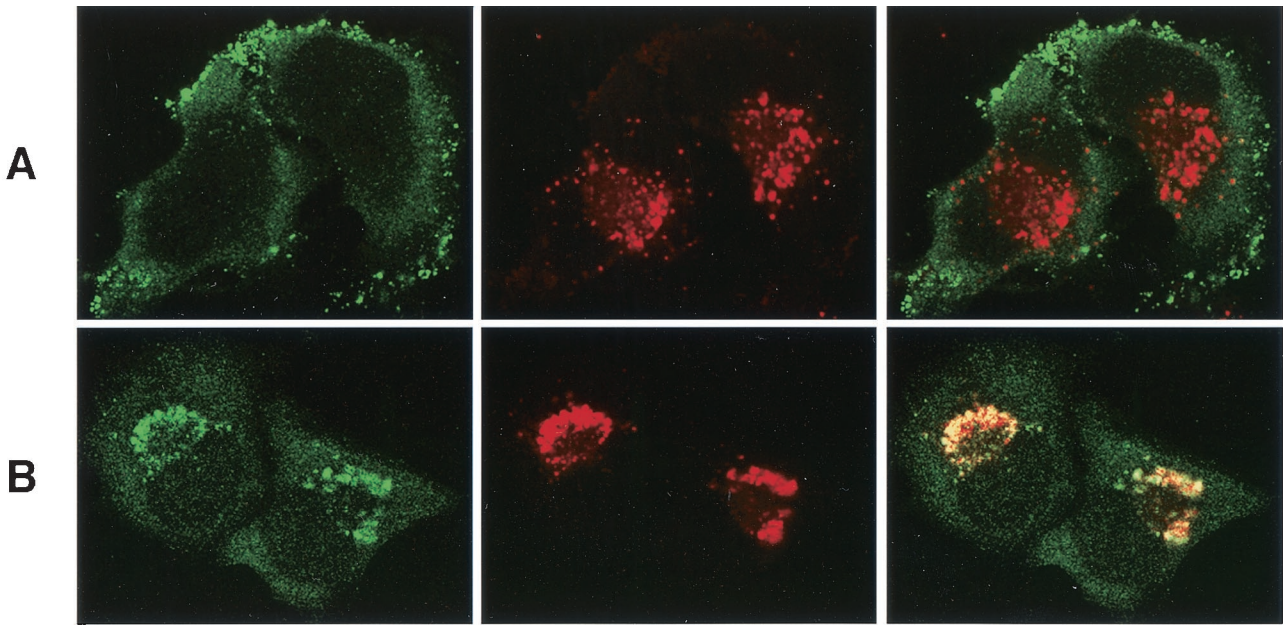


FIG. 2. WT Flag-tagged Gag localizes to the plasma membrane in HeLa cells, but MA mutant Flag-tagged Gag is targeted to the MVB. HeLa cells were transfected with pNL4-3-55FLAG (A) or pNL4-3-55FLAG/29KE/31KE (B) and stained with anti-Flag (left column) or anti-CD63 (middle column). Merged images of Gag and CD63, with colocalization indicated in yellow, are shown in the right column.

and residues 84 to 88 of the MA domain retarget virus assembly to trans- and/or post-Golgi vesicles stained by WGA (41). To define precisely the organelle to which the mutant Gag proteins are retargeted, HeLa cells were transfected with molecular clones encoding WT Gag or mutant Gag proteins containing either 29KE/31KE (41) or 85YG (17) substitutions. Gag-expressing cells were then subjected to double-fluorescence staining by using an anti-MA antibody and various organellar markers. The anti-MA antibody used in this study specifically recognizes the mature form of MA but not the MA domain of unprocessed $Pr55^{Gag}$ (41, 63). Since the majority of Gag processing occurs only after virus particles are formed (11, 55), the MA signal obtained with this antibody most likely represents not just the sites of Gag protein localization but specifically the sites at which virus assembly occurs. Interestingly, the Golgi marker GM130 and the trans-Golgi network marker TGN46 did not colocalize with mutant Gag (Fig. 1A and B). As organelles in the endosomal lineage can be stained by WGA, we also tested several endosomal markers for their colocalization with the mutant Gag proteins. Virtually no colocalization was observed between mutant MA and EEA1, an early endosome marker (43) (Fig. 1C and D). In contrast, a high degree of colocalization was observed between mutant MA and the MVB marker CD63 (Fig. 1E and F). The 29KE/31KE mutant MA also showed partial colocalization with LBPA (Fig. 1G), another marker for the late endosome or MVB compartment (30), and with LysoTracker (Fig. 1H), which visualizes acidic organelles, including late endosomes (or the MVB) and lysosomes. These results suggest that mutations in the highly basic domain or residues 84 to 88 of MA retarget virus particle assembly specifically to the MVB (or a related compartment) instead of the plasma membrane. Importantly, the punctate staining pattern observed for WT Gag at the cell surface only rarely colocalized with CD63 (Fig. 1I). Notably, the results obtained with HeLa cells were recapitulated with Jurkat T cells; the majority of WT Gag was detected on the cell surface and showed minimal overlap with CD63, whereas the majority of the 29KE/31KE mutant Gag signal localized to an intracellular CD63-positive compartment (Fig. 1J and K).

Because the data presented in Fig. 1 were obtained with a monoclonal antibody specific for mature MA, we wanted to examine the localization of unprocessed Gag. Several anti-CA antibodies tested gave a very bright signal for non-membrane-bound, cytosolic Gag; this signal obscured the staining pattern of membrane-bound Gag (data not shown). To circumvent this problem, we expressed a Flag-tagged version of WT $Pr55^{Gag}$ (41) in HeLa cells. When stained with anti-Flag antibody, this full-length Gag displays a plasma membrane punctate staining and a hazy cytosolic staining; the former represents assembled Gag at the plasma membrane, whereas the latter likely represents non-membrane-bound Gag in the cytosol (Fig. 2A). A similar localization was also observed for a WT Gag-green fluorescent protein fusion protein (data not shown). As seen with the mature MA-recognizing antibody (Fig. 1), no significant colocalization was observed between WT Gag and CD63 (Fig. 2A). In rather striking contrast, Flag-tagged Gag containing the 29KE/31KE MA mutation displayed an intracellular distribution pattern that showed considerable colocalization with CD63 (Fig. 2B). Together these results suggest that the retargeted MA mutant Gag traffics to and assembles in the MVB, whereas in HeLa cells transport of WT HIV-1 Gag to an MVB compartment is uncommon.

Both WT and MA mutant Gag proteins are targeted to a CD63-positive organelle in macrophages. Since it has been reported that in macrophages HIV-1 assembly takes place

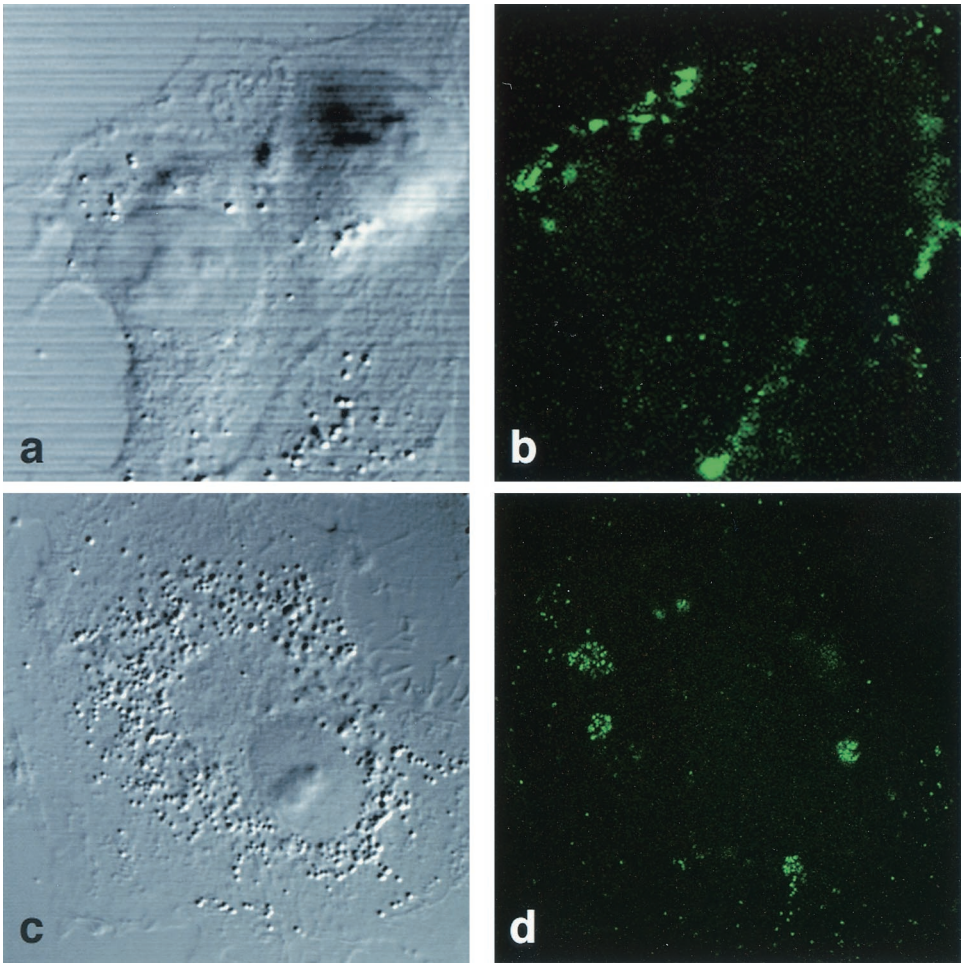
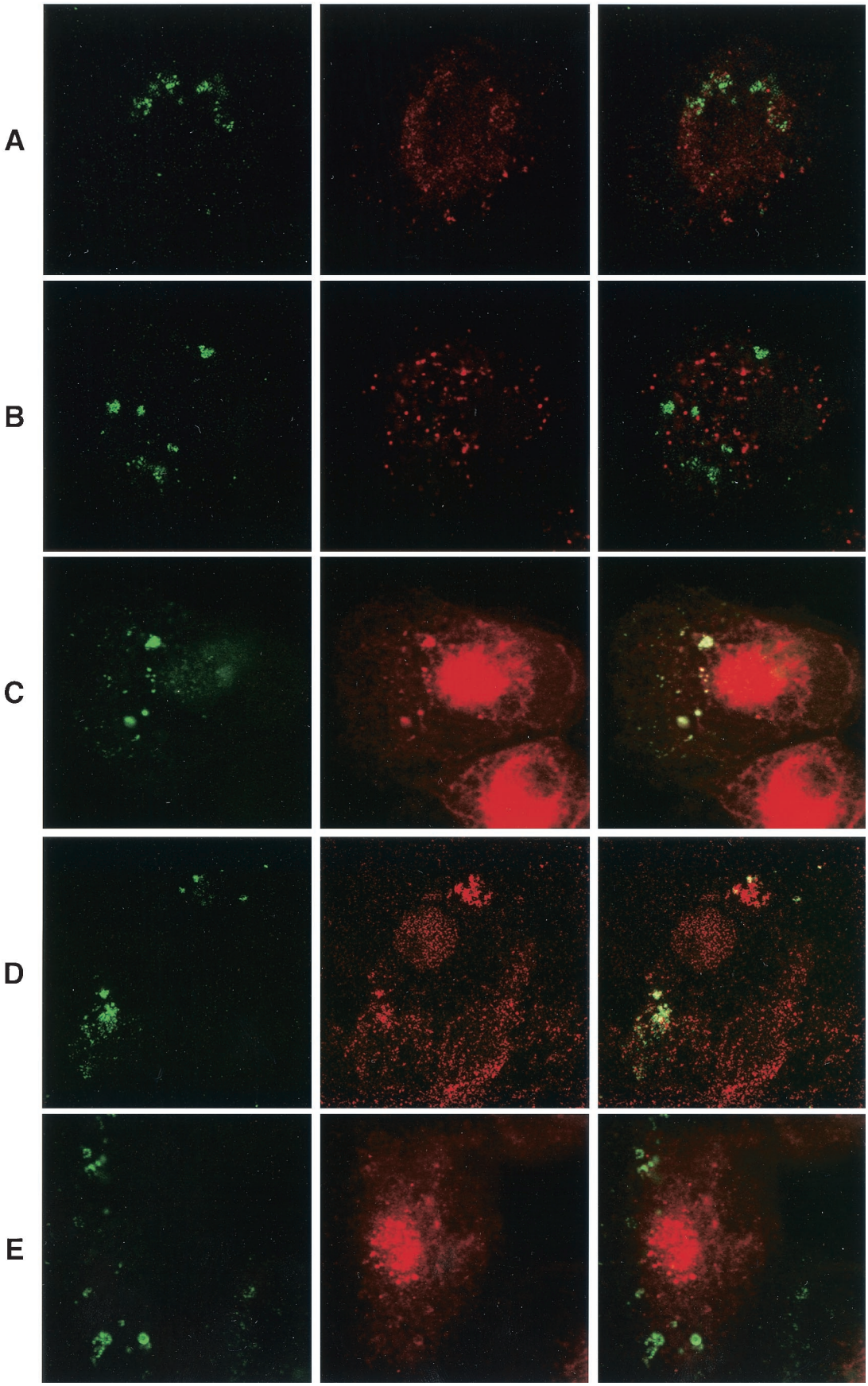


FIG. 3. WT Gag proteins target to an intracellular location in macrophages. HeLa cells (a and b) and monocyte-derived macrophages (c and d) were infected with VSV-G-pseudotyped viruses expressing WT Gag and stained with anti-MA antibody as detailed in Materials and Methods. Differential interference contrast images (a and c) and immunofluorescence images (b and d) are shown.

within intracellular vesicles (42), we sought to compare the targeting phenotypes of WT and mutant Gag proteins in macrophages versus HeLa cells. Monocyte-derived macrophages and HeLa cells were infected with VSV-G-pseudotyped viruses encoding either WT or 29KE/31KE mutant Gag proteins. The localizations of Gag in infected and transfected HeLa cells were similar (compare Fig. 1I and 3b). In contrast to the highly punctate, cell surface staining observed for WT Gag in HeLa cells (Fig. 3a and b), in macrophages the majority of WT MA was detected as a punctate cytoplasmic staining (Fig. 3c and d). The intracellular MA in macrophages did not colocalize with calreticulin, an ER marker, or TGN46 but showed partial colocalization with the post-Golgi marker WGA (data not shown). To identify specifically the organelle to which WT Gag is directed in macrophages, we examined the colocalization of MA with markers for endosomal organelles. EEA1 and internalized fluorescently labeled transferrin did not colocalize with MA (Fig. 4A and B), suggesting that the MA-positive compartment is neither the early nor the recycling endosome. In contrast, MA showed strong colocalization with CD63 (Fig. 4C). As reported previously (1), anti-CD63 staining in macrophages gave two morphologically distinct signals: one bright and perinuclear and the other more peripheral and vesicular. We observed that while the former generally does not colocalize with Gag, the majority of Gag staining colocalized with the latter population of CD63 molecules. Consistent with this finding, Gag also showed a high level of overlap with Rab7 (Fig. 4D), another late endosomal marker (3, 59). Only limited colocalization between MA and LysoTracker was observed (Fig. 4E). These results suggest that WT virus particle assembly in macrophages occurs in a subset of CD63-positive compartments, most likely the MVB or a related organelle. These observations are consistent with recently reported immuno-EM findings (46). Interestingly, macrophages expressing 29KE/31KE or 85YG MA mutant Gag also showed a similar colocalization of MA with CD63 (Fig. 5). These results suggest that although these MA mutations retarget Gag from the plasma membrane to the MVB in HeLa cells, they do not have a significant impact on Gag targeting in macrophages.

Virus particle production in macrophages and HeLa cells takes place with comparable efficiency. The targeting of WT HIV-1 Gag to the MVB in macrophages raised the possibility


A
B
C
D
E

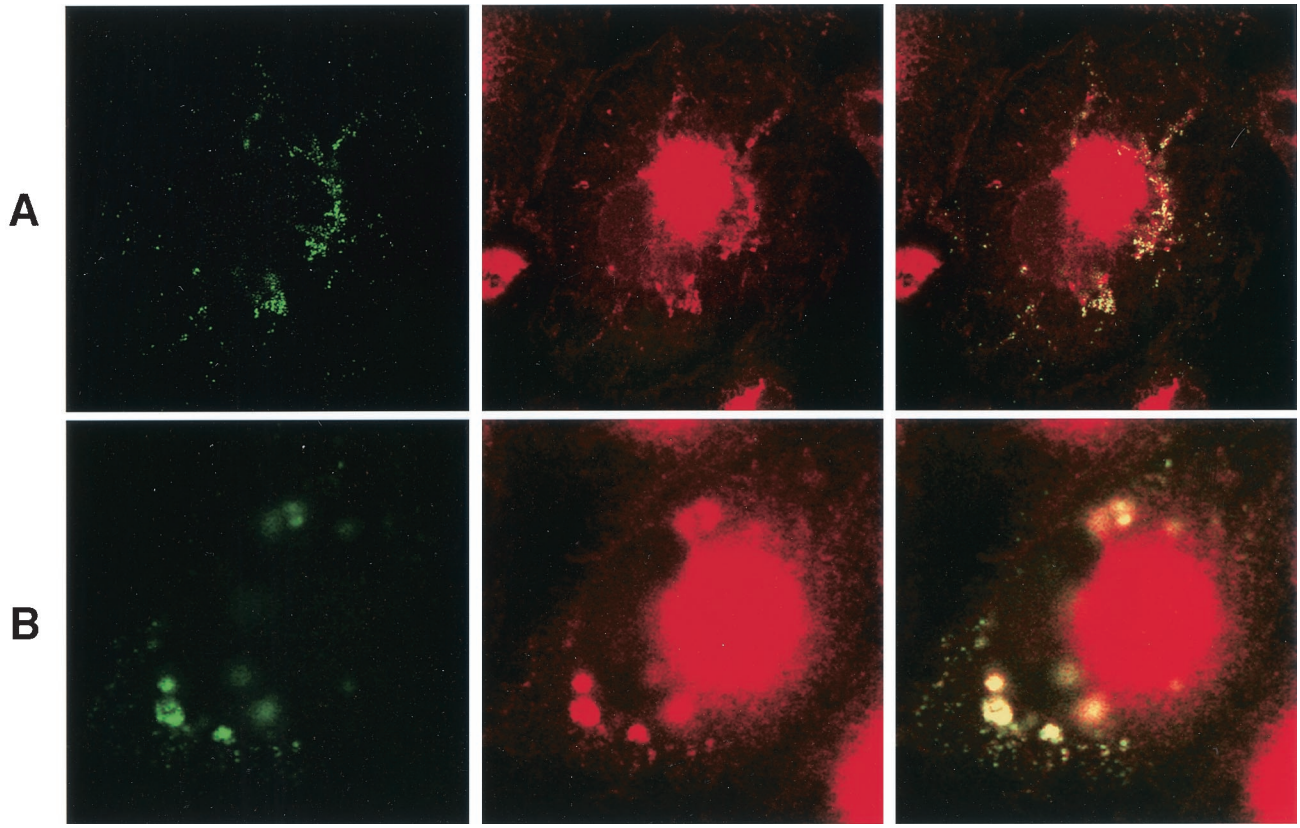


FIG. 5. Mutant Gag proteins specifically localize to a CD63-positive organelle in macrophages. Monocyte-derived macrophages were infected with VSV-G-pseudotyped viruses expressing 29KE/31KE (A) or 85YG (B) mutant Gag proteins and were stained with anti-p17 (left column) and anti-CD63 (middle column) antibodies as detailed in Materials and Methods. Merged images of MA and CD63 signals, with colocalization indicated in yellow, are shown in the right column.

that virus release in this cell type is inherently inefficient, as is the release of MVB-targeted Gag in HeLa cells. To address this possibility, we infected HeLa cells and macrophages with VSV-G-pseudotyped viruses expressing either WT or 29KE/31KE mutant Gag. Infected cells were metabolically labeled with [^{35}S]methionine-cysteine, and cell and virus lysates were subjected to immunoprecipitation with HIV Ig. In macrophages from three different donors, the level of extracellular virus release was quite comparable to or somewhat higher than that observed in HeLa cells (Table 1). These results indicate that Gag trafficking to the MVB in macrophages is not a defective pathway, as it appears to be in HeLa cells, but rather represents part of an efficient route for virus particle release. Consistent with this conclusion, the impact of MA mutations on virus release was less severe in macrophages than in HeLa cells (Table 1).

Targeting of Gag to the MVB is independent of L domain function. Since the L domain present in p6 associates with TSG101 (6, 19, 36, 58; for a review, see references 12 and 45), a protein involved in protein sorting into the MVB (2, 28), it seemed possible that MVB localization could be promoted by the p6-TSG101 interaction. To address this possibility, HeLa cells and macrophages were infected with VSV-G-pseudotyped viruses encoding Gag proteins lacking the entire p6 domain (p6/L1term) (27) and analyzed by confocal microscopy. For Gag targeting to the MVB in HeLa cells, the p6/L1term mutant was compared with full-length Gag in the context of the 85YG MA substitution (the double mutant was designated 85YG/p6$^-$ [41]). HeLa cells expressing 85YG/p6$^-$ Gag showed an accumulation of MA in CD63-positive vesicles (Fig. 6A), as observed for full-length 85YG Gag (Fig. 1F). In macrophages, the involvement of p6 in MVB targeting was assessed in the context of both WT and 85YG MA proteins. Again, the MA signal for the p6/L1term mutant was, like that of WT Gag (Fig. 4C), concentrated in a CD63-positive compartment (Fig. 6B and C). Consistent with these obser-

FIG. 4. WT Gag proteins specifically localize to a CD63-positive organelle in macrophages. Monocyte-derived macrophages were infected with VSV-G-pseudotyped viruses expressing WT Gag and were stained with anti-MA antibody (left column) and a variety of organellar markers (middle column) as detailed in Materials and Methods. The markers used were transferrin (A), anti-EEA1 (B), anti-CD63 (C), anti-Rab7 (D), and LysoTracker (E). Merged images of MA and organellar signals, with colocalization indicated in yellow, are shown in the right column.

TABLE 1. Comparison of efficiencies of WT and mutant virus release from HeLa cells and macrophages

Expt	Cells	Virus release efficiency (% of total)[a]	
		WT	29KE/31KE
1	HeLa cells	32	8
	MDM[b] from donor:		
	A	71	27
	B	66	26
	C	76	43
2	HeLa cells	44	18
	MDM from donor:		
	A	55	46
	B	49	33
3	HeLa cells	28	4
	MDM from donor C	14	12

[a] Calculated as the amount of virion-associated p24 as a fraction of total (cell plus virion) Gag synthesized during a 16-h labeling period.
[b] MDM, monocyte-derived macrophages.

vations, we have observed by EM that intracellular virus particles are still formed in the lumen of membranous organelles in HeLa cells expressing 85YG/p6$^-$ Gag or in macrophages expressing p6/L1term Gag (data not shown) (7, 41). These results indicate that targeting of Gag to the MVB in either HeLa cells or macrophages does not require p6 and is therefore not mediated by a p6-TSG101 interaction.

DISCUSSION

In most cell types such as HeLa cells and T cells, the majority of assembled HIV-1 particles are detected on the plasma membrane. We and others have previously shown (17, 25, 41, 62) that in HeLa and COS cells, amino acid substitutions in the MA basic domain retarget Gag from the plasma membrane to an intracellular organelle. In this study, we identified this intracellular compartment as the MVB (Fig. 1 and 2; summarized in Table 2). The data obtained with T cells essentially recapitulated those derived from HeLa cells (Fig. 1). In contrast to what we observed in HeLa and T cells, in macrophages WT Gag was targeted primarily to the MVB (Fig. 3 and 4; summarized in Table 2) (46). Interestingly, mutations that alter the Gag targeting phenotype in HeLa cells (Fig. 1) (41) did not affect the MVB targeting of Gag in macrophages (Fig. 5). In addition, we determined that virus assembly and release in macrophages are comparably efficient relative to virus particle production in HeLa cells (Table 1), consistent with previous data (50, 51). We also observed that the virus release defect

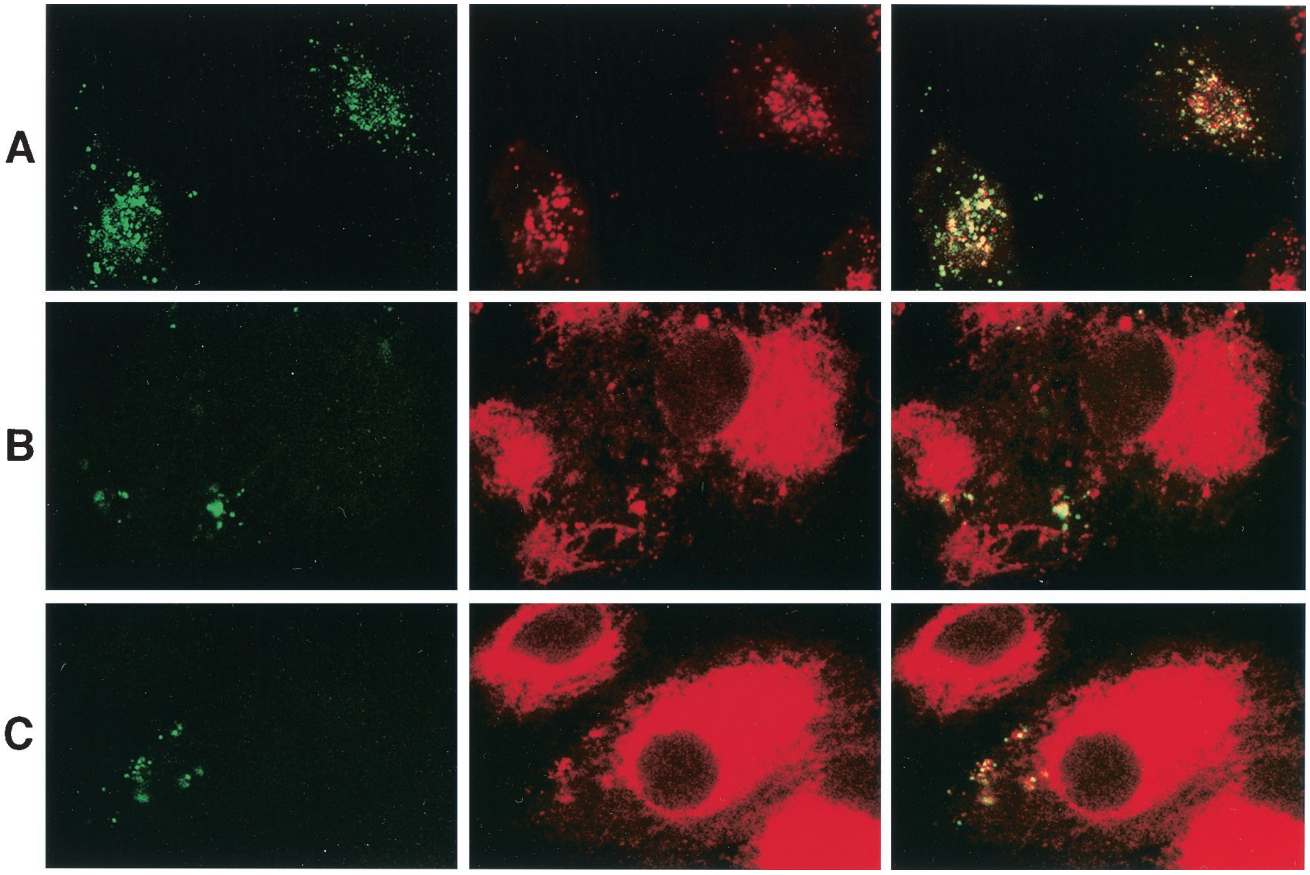


FIG. 6. Gag proteins with p6 deleted still localize to a CD63-positive organelle. HeLa cells (A) and monocyte-derived macrophages (B and C) were infected with VSV-G-pseudotyped viruses expressing 85YG/p6$^-$ (A and C) or p6/L1term (B) mutant Gag proteins and were stained with anti-p17 (left column) and anti-CD63 (middle column) antibodies as detailed in Materials and Methods. Merged images of MA and CD63 signals, with colocalization indicated in yellow, are shown in the right column.

TABLE 2. Colocalization of Gag and organelle markers in HeLa cells and macrophages

Marker (organelle)	Colocalization[a] with:	
	MA mutant Gag in HeLa cells	WT Gag in macrophages
Calreticulin (ER)	−	−
GM130 (Golgi)	−	−
TGN46 (*trans*-Golgi network)	−	−
Transferrin (recycling endosome)	−	−
EEA1 (early endosome)	−	−
CD63 (late endosome or MVB)	+	+
LBPA (late endosome or MVB)	+	NA[b]
Rab7 (late endosome or MVB)	ND[c]	+
LysoTracker (acidic organelles)	+/−	+/−

[a] −, no colocalization; +, significant colocalization; +/−, partial colocalization.
[b] NA, not applicable (LBPA is not detected in macrophages).
[c] ND, not determined.

imposed by the MA mutations analyzed in this study was less severe in macrophages than in HeLa cells (Table 1). Together, these results suggest the presence of an alternative pathway for HIV-1 assembly and release in which Gag is directed to the MVB. In macrophages this MVB targeting leads to efficient virus particle release.

Several lines of evidence argue against the observed localization of Gag to the MVB being a consequence of internalization of released virions. (i) The intracellular Gag signal showed no colocalization with EEA1 or internalized transferrin (Fig. 4), suggesting that Gag proteins are not transported to the MVB from early or recycling endosomes. (ii) Antibody-coated beads phagocytosed by macrophages did not colocalize with Gag for at least several hours (data not shown), suggesting that Gag detected in the MVB did not originate from phagocytosed virus particles. (iii) Cells infected with pseudotyped virus lacking a functional reverse transcriptase failed to show an intracellular Gag signal (data not shown), suggesting that de novo Gag synthesis is required for the observed Gag localization pattern. (iv) EM analyses of macrophages demonstrated that for $p6^{-}$ Gag, virus particles in intracellular vesicles were tethered to the organellar membrane (7). Collectively, these observations strongly suggest that Gag localization to the MVB is the consequence of specific targeting of newly synthesized protein to this intracellular compartment rather than a result of virus internalization from the extracellular space.

Since amino acid substitutions in MA that disrupt plasma membrane targeting in HeLa cells do not cause a promiscuous distribution of Gag to various organelles but instead retarget Gag specifically to the MVB, it is likely that $Pr55^{Gag}$ harbors a distinct MVB targeting signal. It is well established that the L domain sequence in p6 can bind TSG101, a protein that normally functions in the sorting of proteins into the late endosomal pathway (2, 6, 19, 36, 58). However, since removal of p6 altered neither the MVB targeting of MA mutant Gag in HeLa cells nor that of WT Gag in macrophages (Fig. 6), it is unlikely that the interaction between p6 and TSG101 is responsible for recruiting Gag to the MVB. Although substitutions in MA residues critical for HeLa plasma membrane targeting do not alter Gag localization to the MVB in macrophages (Fig. 5), it appears likely that MA contains or influences MVB targeting signals, since large MA deletions either cause Gag to be targeted to the ER or induce a promiscuous Gag distribution (9, 18, 33, 48, 60).

The results obtained in this study could be explained by the existence of two different cellular binding partners for Gag (i.e., Gag receptors), one on the plasma membrane and the other on the MVB. According to this model, in HeLa cells and presumably in T cells, the plasma membrane Gag receptor plays a dominant role if the plasma membrane targeting signal (i.e., the highly basic domain of MA) is intact. If this signal is altered, Gag would bind the MVB receptor and form virus particles in the MVB. As a result, the efficiency of virus particle release from the cell would be reduced. In contrast, according to this model, in macrophages the MVB receptor is dominant. Consequently, Gag proteins, regardless of the presence of an intact plasma membrane targeting domain, would form virus particles in the lumen of the MVB. The identities of the putative Gag receptors on the plasma membrane and the MVB membrane remain to be defined. Gag receptors could be proteinaceous or lipidic in nature. It has been reported that replacement of the Gag N-terminal myristate with a polyunsaturated fatty acid reduces the affinity of Gag for lipid rafts and causes mistargeting in COS cells (34). It is therefore possible that lipid rafts in the plasma membrane promote Gag targeting to the cell surface. However, although depletion of cellular cholesterol disrupts lipid rafts and impairs virus production (38), it does not appear to alter Gag targeting in HeLa cells (A. Ono and E. O. Freed, unpublished data). Interestingly, a variety of proteins are known to be targeted to the plasma or endosomal membranes through specific interactions with phosphoinositides, raising the possibility that these lipids may play a role in Gag targeting (56, 57). Studies to define further the cellular and viral determinants of Gag targeting to the plasma membrane and the MVB are under way.

It has been observed that in a variety of hematopoietic cell types, MVBs can release their contents to the extracellular space via the so-called exosomal pathway. Exosomal release takes place when MVBs traffic to the cell surface and fuse with the plasma membrane (54). Thus, in contrast to MVB targeting in HeLa cells, which results in virus retention, particles formed in the MVB in macrophages would potentially be released via the exosome pathway, consistent with immuno-EM observation of HIV-1-infected macrophages (46). Our finding that the process of virus assembly and release in macrophages is relatively efficient supports the idea that Gag targeting to the MVB constitutes a physiologically relevant process for virus particle production in this cell type. While this paper was under review, Pelchen-Matthews et al. published a study in which immuno-EM and characterization of virus-incorporated cellular proteins were used to support a model for MVB assembly of HIV-1 in macrophages (44). Interestingly, viruses from several different families (e.g., *Filoviridae* and *Herpesviridae*) have also been observed to assemble in the MVB (10, 32). Since a budding process topologically identical to that of enveloped viruses takes place in MVBs, targeting of virus assembly to the MVB may represent a general process for enveloped virus release from cell types in which the exosomal pathway is active.

ACKNOWLEDGMENTS

We thank D. Demirov and M. Xhilaga for critical review of the manuscript and S. Ablan for expert technical assistance. We thank O. Schwartz for assistance with confocal microscopy and A. Buckler-White for sequencing. We thank K. Fields and K. Clouse-Strebel for the monocytes, J. Burns and D. Rekosh for the plasmids, and J. Gruenberg for the anti-LBPA antibody. HIV Ig was obtained through the NIH AIDS Research and Reference Reagent Program.

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