degradation of ferroportin, a cell surface receptor that is downregulated by hepcidin binding (55), shows a strong dependence on TSG101, EAP20, and CHMP6 (I. De Domenico, D. M. Ward, C. Langelier, M. B. Vaughn, E. Nemeth, W. I. Sundquist, T. Ganz, G. Musci, and J. Kaplan, unpublished data). We therefore conclude that lysosomal targeting of different cell surface receptors can differ in EAP20 and CHMP6 requirements. Some receptors, such as MHC-I, exhibit little or no requirement for these proteins, while other receptors, such as ferroportin, exhibit strong requirements, and still others, such as EGFR, exhibit intermediate phenotypes. These experiments indicate that there are probably multiple pathways for sorting membrane proteins into the MVB vesicles of mammalian cells.

DISCUSSION

Not surprisingly, our studies have revealed that the human ESCRT-II complex has a number of features in common with its better-characterized yeast counterpart. The similarities include interactions with ESCRT-I, ESCRT-III, and ubiquitin; transient association with endosomal membranes; and functional participation in receptor downregulation. These observations are all consistent with the idea that ESCRT-II can play a central role in the human ESCRT pathway, interacting with the upstream ESCRT-I and downstream ESCRT-III complexes and with ubiquitylated cargos as they are sorted into MVB vesicles. Nevertheless, depletion of EAP20/ESCRT-II and CHMP6/ESCRT-III did not significantly reduce HIV-1 release from cultured 293T or HeLa cells, indicating that only a subset of the mammalian ESCRT machinery is required for efficient virus release from these cell types.

Role of EAP20/ESCRT-II in HIV-1 release. We considered several possible explanations for the observation that EAP20/ESCRT-II depletion does not inhibit HIV-1 release. As with all siRNA depletion experiments, it is possible that even the very low levels of EAP20 remaining after depletion were still sufficient to function in HIV-1 release (and MHC-I downregulation). While we cannot rule out this possible explanation, it seems unlikely because EAP20 was efficiently depleted in our experiments (Fig. 7 and 10), and this depletion measurably inhibited EGFR degradation and lysosomal targeting (Fig. 9 and 10). Furthermore, EAP20 depletion actually produced a slight, but reproducible, increase in HIV-1 infectivity (Fig. 7), which could be explained if the endogenous ESCRT-II complex binds and competes for other factors essential for virus budding (e.g., ESCRT-I). It is also conceivable that ESCRT-II may remain active even without EAP20 subunits. This explanation again seems unlikely, because the two copies of EAP20 comprise half of the ESCRT-II subunits and form the only known link between ESCRT-II and ESCRT-III. Furthermore, ESCRT-II activity is blocked by deletion of EAP20/Vps25p in both *S. cerevisiae* and *Drosophila* (4, 76, 77). We therefore conclude that the most likely explanation for our data is that ESCRT-II is not required for efficient HIV-1 release from human cells, either because the complex does not normally participate in virus budding or because the viral budding machinery still functions efficiently in its absence.

Our results also imply that if HIV-1 utilizes the late-acting ESCRT-III and VPS4 complexes to bud from cells, then there must be other proteins or complexes, in addition to ESCRT-II, that can bridge ESCRT-I and ESCRT-III. In principle, ALIX/AIP1 could be one such bridging protein, as it can bind directly to both TSG101 (83) and to the CHMP4 proteins (38, 41, 69, 83), although simultaneous depletion of ALIX and EAP20 did not synergistically inhibit EGFR degradation (11). Similarly, interactions between the yeast ESCRT-I components, Vps37p and Vps28p, and the ESCRT-III component, Vps20p (CHMP6), have been reported (10), although we did not observe analogous ESCRT-I–ESCRT-III interactions between the isolated human proteins in two-hybrid experiments. Finally, the deubiquitylating enzyme, AMSH, binds both the upstream HRS-STAM complex (which binds to TSG101) (71) and the ESCRT-III components CHMP3 and CHMP4 (23). In principle, these (and other) bridging interactions could help to recruit ESCRT-III to sites of MVB vesicle formation and virus budding even in the absence of ESCRT-II.

Role of ESCRT-III in HIV-1 release. Models for the roles of ESCRT-III (and VPS4) in virus budding must take into account the observation that HIV-1 release is not significantly inhibited by depletion of endogenous CHMP5 and CHMP6 (reference 85 and this work), yet virus release is very potently inhibited by overexpression of dominant-negative CHMP5 or CHMP6 protein (46, 69, 70). siRNA depletion of these individual CHMP proteins therefore imposes a less severe (or less general) block in the ESCRT pathway than the dominant-negative ESCRT-III and VPS4 constructs. A likely explanation for this difference is that dominant-negative CHMP (and VPS4) constructs function by inducing the formation of aberrant endosomal compartments (class E compartments) that not only inhibit the functions of the modified CHMP proteins themselves, but also sequester other ESCRT complexes and possibly many other MVB proteins required for HIV-1 budding. Thus, we cannot rule out the possibility that wild-type CHMP and VPS4 proteins do not normally function in HIV-1 release but rather affect virus budding only when present in dominant-negative forms. However, we favor a model in which at least a subset of the ESCRT-III and VPS4 proteins do actually participate directly in virus release but where depletion of individual CHMP5 or CHMP6 proteins does not inhibit virus budding owing to (i) functional redundancy within the MVB pathway arising from the presence of 10 different CHMP proteins, (ii) the possibility that CHMP5 (and possibly other CHMP proteins) may function at a step following MVB vesicle formation (7, 66), and/or (iii) the presence of multiple distinct ESCRT pathways in human cells. It therefore continues to be important to determine precisely which subset of different ESCRT proteins is utilized directly by HIV-1 and other viruses.

Complexity of MVB vesicle formation and protein sorting in higher eukaryotes. Genetic studies have identified the basic machinery required for MVB vesicle formation and protein sorting in *S. cerevisiae*. Importantly, deletion of each of the six known yeast ESCRT-I and ESCRT-II subunits ultimately leads to a strong block in protein sorting and MVB vesicle formation (the class E phenotype) (40). While it seems almost certain that the basic mechanism of MVB biogenesis is highly conserved, because human cells have at least one ortholog of every known yeast class E protein (33, 51), our studies add to the growing number of indications that MVB protein-sorting/vesicle formation pathways are more complex in higher eu-