complex is not absolutely required for membrane protein sorting or MVB vesicle formation (11). Indeed, several groups have proposed that ESCRT-II complexes in other species may perform functions that appear to be unrelated to MVB protein sorting. These include a role for mammalian ESCRT-II in transcriptional elongation (37, 61, 65) and a role for the *Schizosaccharomyces pombe* ESCRT-II protein EAP30 in meiotic spindle pole body amplification (35). The relationship between MVB protein sorting and these other putative ESCRT-II activities, if any, is not yet clear.

During HIV-1 assembly, human class E proteins are recruited by two short sequence motifs (termed "late domains") within the C-terminal p6 region of the Gag polyprotein (reviewed in references 15, 19, 22, 47, 48, 52, 70, and 79). The first $p6^{gag}$ late domain is a PTAP tetrapeptide motif that binds and recruits TSG101 (*t*umor *s*usceptibility *g*ene 101; yeast Vps23p). TSG101 is the central protein of the human ESCRT-I complex, and there is good evidence that the entire ESCRT-I complex functions directly in HIV-1 release (15, 19, 22, 24, 48). The second late domain within $p6^{gag}$ is a YPX_nL motif (where X_n can vary in identity and length) that binds and recruits ALIX/AIP1 (yeast Bro1p) (69, 80). ALIX functions late in MVB biogenesis (56) and binds both ESCRT-I (83) and ESCRT-III (41) proteins but is not a permanent subunit of any of the three discrete ESCRT complexes. Like TSG101, ALIX functions directly in virus release, although disruptions of the PTAP-TSG101 interaction generally inhibit HIV-1 budding to a greater degree than do disruptions of the ALIX-YPX_nL interaction (16, 46). As noted above, there are also indications that HIV-1 budding requires the activities of the late-acting ESCRT-III, LIP5, and VPS4 class E proteins (22, 46, 69, 83, 85). Specifically, HIV-1 release is dominantly inhibited by overexpression of VPS4 ATPase mutants and ESCRT-III proteins with large N- or C-terminal extensions (22, 46, 69, 83). HIV-1 release is also impaired by small interfering RNA (siRNA) depletion of the VPS4 binding protein LIP5 (85). It is important to note, however, that these experiments do not definitively establish a direct role for these proteins in HIV-1 budding, because the inhibitory effects could also arise from sequestration of the entire ESCRT machinery at aberrant endosomal compartments (called "class E" compartments).

In summary, HIV-1 uses the ESCRT-I complex to bud from cells and also requires the activities of downstream ESCRT-III and VPS4-LIP5 complexes. In *S. cerevisiae*, ESCRT-II physically bridges the ESCRT-I and ESCRT-III complexes and is essential for MVB protein sorting and vesicle formation (4). The mammalian ESCRT-II function appears to be more complex, however, and we therefore undertook this study with the goals of characterizing the biochemical properties of human ESCRT-II and testing its role in HIV release.

MATERIALS AND METHODS

ESCRT-II homology modeling. A working model for the human ESCRT-II core was created as follows. Protein Data Bank (PDB) coordinates from a yeast ESCRT-II crystal structure (PDB accession no. 1U5T) were utilized to create a consensus template using Swiss-PdbViewer (http://www.expasy.org/spdbv/). The human and yeast ESCRT-II sequences were then aligned within Swiss-PdbViewer, the alignment was used to thread the human sequence onto the yeast ESCRT-II template, and the model was energy minimized within Swiss-PdbViewer.

Plasmids. Genes for human EAP20, EAP30, EAP45, and CHMP6 used in yeast two-hybrid and biochemical experiments were amplified from expressed sequence tag clones as described previously (83). A complete list of all constructs used in this study is provided in Table S1 in the supplemental material. Genes and fragments for yeast two-hybrid experiments were cloned into pGADT7 (activation domain) and pGBKT7 (binding domain) vectors (Clontech) using 5′ NdeI and 3′ BamHI or BglII cloning sites. Recombinant proteins used in biochemical experiments were expressed as glutathione *S*-transferase (GST) C-terminal fusions from a modified pGEX vector (GE Biosciences). This vector (pGEX2T-TEV) contained a tobacco etch virus (TEV) protease cleavage site and 5′ NdeI and 3′ BamHI or BglII cloning sites following the GST gene. Expression constructs for deletion mutants were created by amplifying and subcloning the desired fragments or by introducing a stop codon using Quickchange mutagenesis (Stratagene). For mammalian expression, ESCRT genes were cloned as EcoRI-BamHI fragments into pcDNA3.1(−)MycHis in frame with the C-terminal Myc tag, and with a Kozak sequence, ACC, inserted between the EcoRI site and the ATG start codon.

Preparation of recombinant EAP20. GST-EAP20 was expressed in *Escherichia coli* BL21(DE3) transformed with WISP05-68 (see Table S1 in the supplemental material) by induction in mid-log phase (0.4 mM IPTG [isopropyl-β-D-thiogalactopyranoside]) and allowing the protein to accumulate for 4 hours at 23°C. The cells were lysed by lysozyme treatment (2.5 mg/ml) in 50 mM Tris, pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), followed by sonication and centrifugation (at 27,000 × *g* for 45 min) to remove cell debris. Soluble GST-EAP20 was bound to a glutathione-Sepharose affinity matrix (GSTPrep FF; GE Healthcare), washed, and eluted with a step gradient of 20 mM glutathione. Protein fractions were dialyzed into 25 mM Tris (pH 8.0), 150 mM NaCl, and 1 mM DTT), and the fusion protein was cleaved during dialysis with 1 mg TEV protease per 100 mg protein (24 h; 23°C). The TEV cleavage reaction mixture was adjusted to 25 mM Tris (pH 8.0), 300 mM NaCl, 10% glycerol, 1 mM DTT, and free EAP20 was separated from GST and GST-EAP20 by size exclusion chromatography (Superdex 200; GE Healthcare). This purification method typically yielded ~1.5 mg of pure protein per liter of starting culture.

Preparation of recombinant CHMP6 proteins. GST-CHMP6 expression, affinity purification, and TEV cleavage were carried out as described for GST-EAP20. Following TEV cleavage, free GST was removed by glutathione-Sepharose affinity chromatography. The solution was then diluted six-fold to reduce the salt in the buffer to 50 mM NaCl, and the CHMP6 protein was purified to homogeneity by anion-exchange chromatography on immobilized Q-Sepharose (GE Biosciences). CHMP6 eluted at ~400 mM NaCl from a linear gradient of 50 mM to 1 M NaCl in 25 mM Tris (pH 8.0), 10% glycerol, 1 mM DTT.

GST pull-down assays. GST and GST-CHMP6 fusion proteins were expressed in *E. coli* BL21(DE3) and induced at mid-log phase with 0.4 mM IPTG. Ten-milliliter cultures of cells expressing GST-CHMP6 were pelleted; resuspended in 2 ml of 300 mM NaCl, 0.1% NP-40, 10% glycerol, 1 mM DTT, 50 mM Tris (pH 8.0); and lysed by lysozyme treatment and sonication. Soluble GST-CHMP6 was incubated with 100 μl glutathione-Sepharose resin. Approximately 30 μM purified recombinant EAP20 was added to 2 μM (equivalent) of immobilized GST-CHMP6 and incubated for 30 min at 4°C in resuspension buffer. Unbound EAP20 was removed by washing with lysis buffer, and bound proteins were released from the resin by boiling it in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and were analyzed on Coomassie-stained 4 to 20% SDS-PAGE gradient gels.

Yeast two-hybrid binding assays. Directed yeast two-hybrid assays were performed using the Matchmaker GAL4 Yeast Two Hybrid 3 system (Clontech). Briefly, *Saccharomyces cerevisiae* AH-109 was cotransformed with pGADT7 or pGBKT7 cloning vector (Clontech) containing the inserts of interest. The transformed yeast colonies were grown for 3 days at 30°C on yeast extract-peptone-dextrose plates with minus Leu, minus Trp selection. Ten to 100 colonies were pooled, resuspended in a liquid culture of Sabourand dextrose broth (minus Leu, minus Trp), selected on Sabourand dextrose broth (minus Leu, minus Trp, minus Ade, minus His) plates, and allowed to grow for 3 days.

Antibody production. Antibodies against the purified recombinant proteins EAP20 (UT461 and -462) and CHMP6 (UT463 and -464) were raised in rabbits by Covance Inc., following their 87-day protocol (http://abservices.crpinc.com/ab_sampleProtocols.aspx). UT461 and UT463 were affinity purified with the respective proteins, as described previously (83).

Biosensor binding experiments. EAP45-ubiquitin biosensor binding experiments were performed at 20°C using a BIACORE 2000 (Biacore AB, Uppsala, Sweden) equipped with a research grade CM4 sensor chip. Approximately 5,000 response units (RU) anti-GST antibody was immobilized on all four flow cells using amine-coupling chemistry (36). GST-$EAP45_{1–229}$, GST-$EAP45_{1–139}$, or recombinant GST alone from soluble *E. coli* lysates was diluted in running buffer