

## Cellular Motor Protein KIF-4 Associates with Retroviral Gag

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Previously we demonstrated that murine retroviral Gag proteins associate with a cellular motor protein, KIF-4. Using the yeast two-hybrid assay, we also found an association of KIF-4 with Gag proteins of Mason-Pfizer monkey virus (MPMV), simian immunodeficiency virus (SIV), and human immunodeficiency virus type 1 (HIV-1). Studies performed with mammalian cell systems confirmed that the HIV-1 Gag protein associates with KIF-4. Soluble cytoplasmic proteins from cells infected with recombinant vaccinia virus expressing the entire Gag-Pol precursor protein of HIV-1 or transfected with HIV-1 molecular clone pNL4-3 were fractionated by sucrose gradient centrifugation and further separated by size-exclusion and anion-exchange chromatographies. KIF-4 and HIV-1 Gag cofractionated in both chromatographic separations. Immunoprecipitation assays have also verified the KIF-4-Gag association. KIF-4 binds mainly to the Gag precursor (Pr55 Gag) and a matrix-capsid processing intermediate (Pr42) but not to other processed Gag products. The binding of Gag is mediated by a domain of KIF-4 proximal to the C terminus. These results, and our previous studies, raise the possibility that KIF-4 may play an important role in retrovirus Gag protein transport.

A key step in the retroviral replication cycle involves a process in which a large number of chemically distinct macromolecules are transported through different pathways to the plasma membrane of the cell, where they are assembled into nascent viral particles. The internal protein shell or capsid of the virus is assembled from a large number of polyprotein precursors that must be transported through the cytoplasm—either preassembled, in small groups, or as monomers—to the inner face of the plasma membrane. The Gag protein of retroviruses directs the assembly and release of virus-like particles from the cell even when expressed in the absence of all other virus-encoded components (12). Several mechanisms of Gag transport have been suggested: (i) free diffusion from the cytoplasm to the cell membrane, (ii) interaction with elements of the cytoskeletal system (2), or (iii) binding to the outer surface of the Golgi apparatus and then trafficking to the plasma mem-

brane on transport vesicles (7). The actual mechanism by which Gag polyproteins arrive at the plasma membrane remains unclear.

KIF-4 was identified as a cellular motor protein that belongs

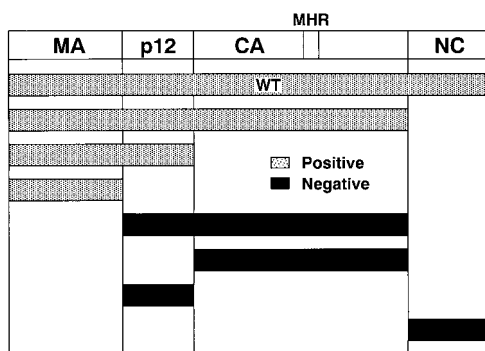


FIG. 1. Binding domain for KIF-4 in MMLV Gag in YTHS. Gal4AD-Y26 was used as bait to interact against different Gag domains fused with Gal4DB. CA, capsid; NC, nucleocapsid; WT, wild-type; MHR, major homology region.

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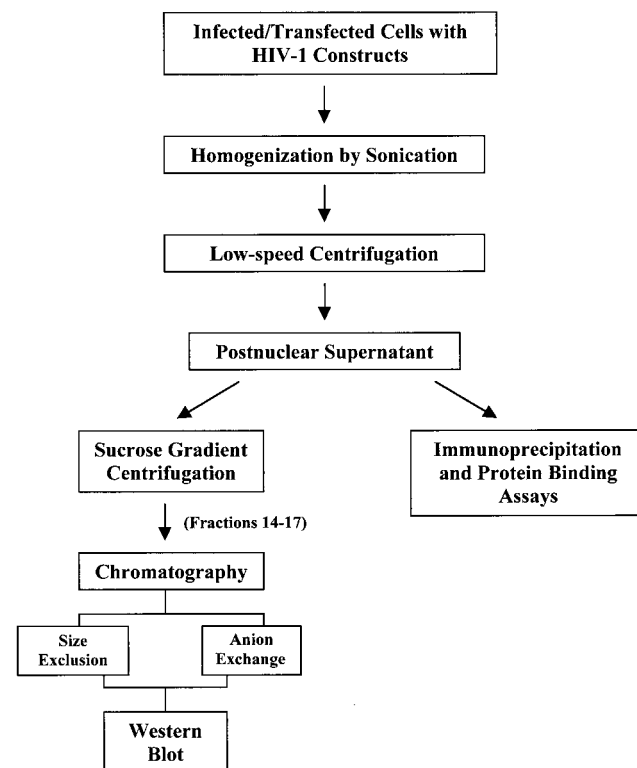


FIG. 2. Flow chart for the identification of KIF-4-HIV-1 Gag interaction in vVK-infected HeLa cells.

TABLE 1. Interactions between KIF-4 and Gag proteins

Virus and Gag protein	Interaction with KIF-4 <sup>a</sup>
RSV Pr65 <sup>Gag</sup> .....	++
MMLV Pr65 <sup>Gag</sup> .....	++
MPMV Pr76 <sup>Gag</sup> .....	++
SIV Pr57 <sup>Gag</sup> .....	++
HIV-1 Pr55 <sup>Gag</sup> .....	++
No DNA.....	-

<sup>a</sup> ++, strongly reactive; -, nonreactive.

to the kinesin superfamily (11). Structurally, it has three domains: an NH<sub>2</sub>-terminal globular motor, a central α-helical stalk, and the COOH-terminal tail. This protein is expressed in all cell lines and mouse tissues tested (6). The intracellular localization of KIF-4 as determined by immunocytochemistry and subcellular fractionation suggests that nearly 50% of KIF-4 is located in the nucleus, while the rest associates with membranous organelles (11). Recent studies have demonstrated that about 25% of cytoplasmic KIF-4 is associated with mitochondria in human immunodeficiency virus type 1 (HIV-1)-transfected cell lines (13). It has been suggested that KIF-4 is a microtubule plus end-directed motor for transport of a cer-

tain group of membranous organelles in juvenile neurons and other cells (11). The function of KIF-4, however, is not fully understood.

Our previous studies showed that a C-terminal portion of KIF-4, designated Y26, interacts with murine leukemia virus (MuLV) Gag proteins in the yeast two-hybrid system (YTHS) and in MuLV-infected cells (6). Here we show that this domain also binds to HIV-1, simian immunodeficiency virus (SIV), Mason-Pfizer monkey virus (MPMV), and Rous sarcoma virus (RSV) Gag proteins in the YTHS and present detailed studies of the KIF-4-HIV-1 Gag association. The data suggest that KIF-4 may play a role as a molecular motor for Gag transport.

**KIF-4 binds multiple retrovirus Gag polyproteins in the YTHS.** The C-terminal portion of KIF-4, isolated previously as a Gal4AD fusion clone (designated Gal4AD-Y26), was used as bait to interact against multiple Gag polyproteins, including those of HIV-1, SIV, MPMV, RSV, and Moloney MuLV (MMLV). The respective gag genes were cloned into pMA424 and fused with the Gal4 DNA-binding domain (Gal4DBD-Gag) for analysis in the YTHS as described before (1, 3, 9). We found that KIF-4 interacted strongly with all full-length Gag proteins (Table 1). This result confirms and extends our previous studies that showed that KIF-4-Gag association could be detected by coimmunoprecipitation in cells infected with dif-

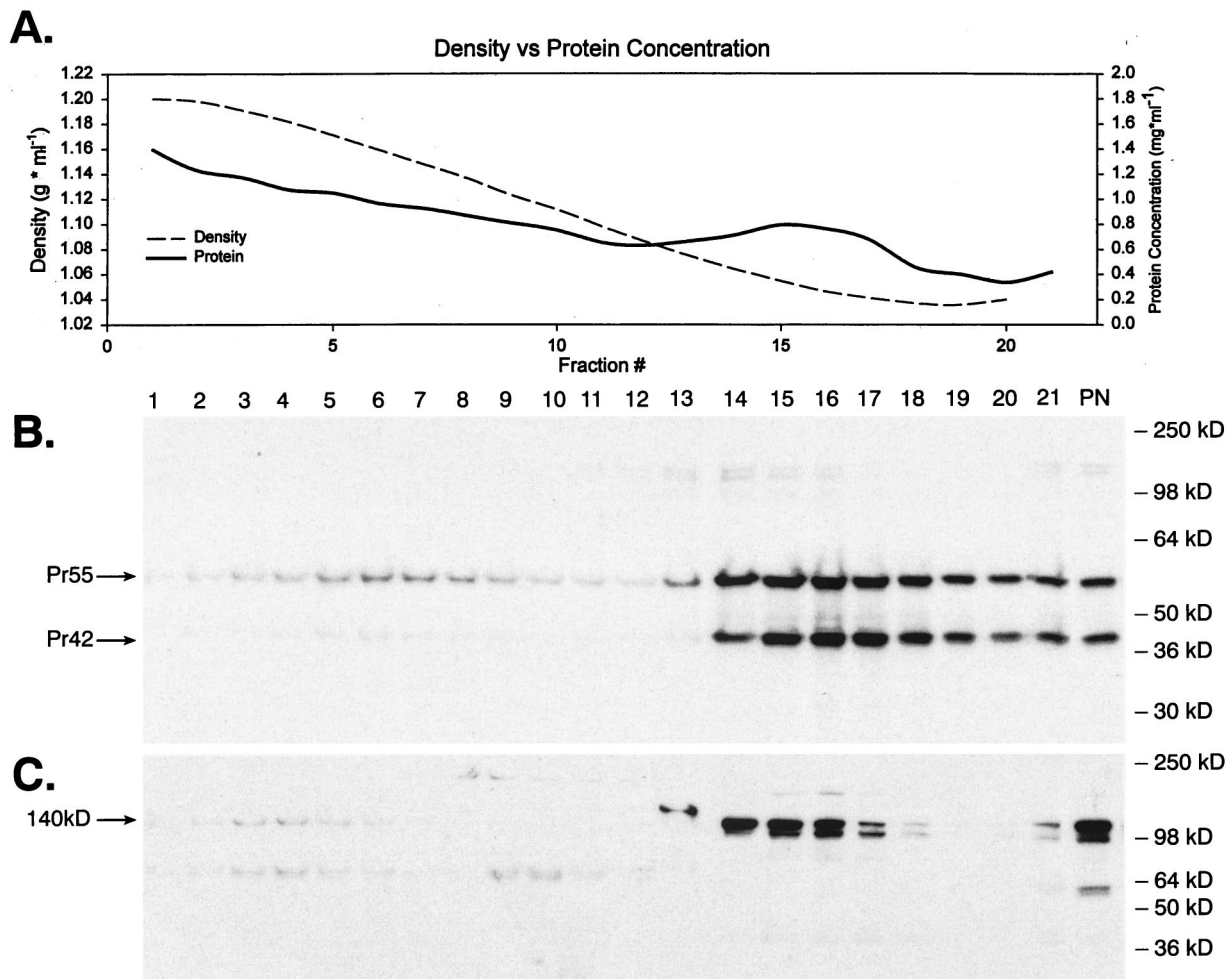


FIG. 3. Sucrose gradient fractionation of postnuclear supernatants from vVK-infected HeLa cells. (A) Protein density versus total protein concentration; (B and C) Western blotting of each fraction with anti-HIV-1 Gag Pr24 (B) and anti-KIF-4 (C). PN, postnuclear cell lysate.

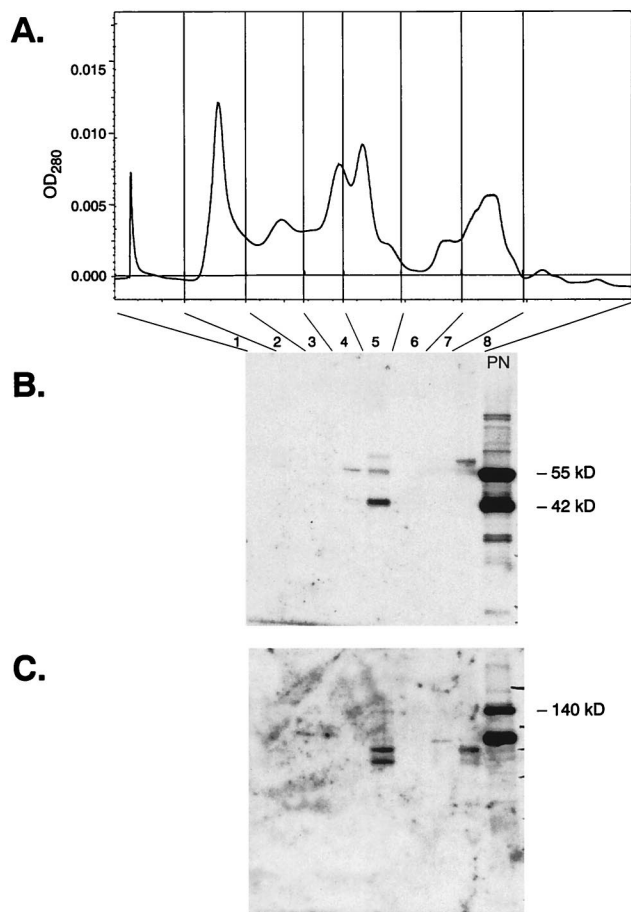


FIG. 4. Size-exclusion chromatography analysis of vVK-infected HeLa cells. Fractions 14 to 17 from sucrose gradient separation were pooled and passed through a 1000/17 column. (A) Histogram of protein distribution in each fraction and grouping of fractions according to elution peaks of protein; (B and C) Western blot analysis of each group with anti-HIV-1 Gag Pr24 monoclonal antibody (B) and anti-KIF-4 antibody (C). PN, postnuclear cell lysate; OD<sub>280</sub>, optical density at 280 nm.

ferent classes of MuLV (6). To map the binding site of KIF-4 to Gag, MMLV Gag fragments were amplified by PCR as a single domain or as combinations of domains (Fig. 1) and cloned into the pSH2-1 vector encoding fusion with the Lex-ADBD binding domain. We found that the KIF-4 binding site of MMLV Gag is located in matrix (MA) (Fig. 1). An identical result was obtained when pGBT9 was used as a vector for Gag domain fusion constructs (data not shown).

The MA domain of Gag plays a crucial role in retrovirus assembly and budding by virtue of myristylation and resulting plasma membrane association, although myristylation itself is not sufficient for plasma membrane binding (4). It is not clear when Gag binds to the cytoplasmic membrane, but the finding that MA associates with KIF-4 could point to a mechanism for Gag polyprotein transport from the cytoplasm to the inner face of the cell membrane.

**Identification and characterization of KIF-4-HIV-1 Gag association in vivo.** The association of KIF-4 and HIV-1 Gag proteins was examined further by using the procedures outlined in the flow chart shown in Fig. 2. HeLa cells ( $5 \times 10^6$ /75-cm<sup>2</sup> flask) were plated and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) (Quality Biological, Inc., Gaithersburg, Md.) with 5% fetal bovine serum. The next day,

the cells were washed twice with serum-free medium and infected with the HIV-1-expressing vaccinia virus vector vVK (5), kindly provided by Bernard Moss (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Disease, National Institutes of Health). The vVK virus stock was diluted in serum-free medium to a concentration of 0.02 PFU/cell and incubated with HeLa cells at 37°C for 1 h. The cells were then cultured in 5% fetal bovine serum-DMEM medium (without washing away the virus) for another 48 to 72 h for maximum cytopathic effect. The cells were harvested by gently scraping them from the flask, washed twice in H-S buffer (10 mM HEPES, 0.5 M NaCl [pH 7.2]), and then resuspended in 2 ml of H-S buffer containing 6% sucrose and protease inhibitors (COMPLETE Mini; Boehringer Mannheim, Indianapolis, Ind.). Sonication was performed in a tank-type sonicator for 10 s/run until more than 80% of the cells were broken as determined microscopically. Unbroken cells and nuclei were removed by centrifugation at  $1,200 \times g$  for 5 min to obtain a postnuclear fraction. All procedures were performed on ice or at 4°C to prevent proteolysis.

The postnuclear fraction (2 ml) was loaded onto a sucrose equilibrium-density gradient, which was prepared by layering 1.5 ml of each stock sucrose solution (from 60 to 10%, bottom to top, in 10% increments) and equilibration for 24 h at 4°C. The gradients were then ultracentrifuged in an SW41 rotor (Beckman, Columbia, Md.) at 35,000 rpm for 22 h at 4°C. Fractions (0.5 ml) were aspirated from the bottom to the top. Protein concentrations were measured with a bicinchoninic acid protein detection kit (Pierce, Rockford, Ill.) (Fig. 3A). Western blot analyses, performed as described previously (6), were used to detect fractions containing KIF-4 and HIV-1 Gag. Polyclonal anti-KIF4 (11) and monoclonal anti-HIV-1 P24 antibodies (NIH AIDS Research and Reference Reagent Program, Rockville, Md.) were the detecting reagents. Among the 21 fractions, most of HIV-1 Gag protein was seen in fractions 14 to 21, with a peak at fractions 14 to 17 in the density range of 1.05 to 1.08 (Fig. 3B). The Gag-Pol Pr160 was seen in fractions 13 to 16, colocalized with the peak of Pr55 and Pr42 (MA-capsid). A small amount of Gag was also detected in fractions 4 to 8 (density, 1.15 to 1.17) with Gag precursor Pr55 and small amounts of Pr42 and P24 (data not shown). The full-length KIF-4 protein (140 kDa) colocalized with Pr55 in fractions 14 to 17 (Fig. 3C).

A recent study described two forms of putative Gag assembly intermediates in HIV-1-infected CD4<sup>+</sup> T cells, HIV-1-infected SupT-1 and Jurkat cells, and HIV-1-transfected HeLa and COS cells—a detergent-resistant complex and a detergent-sensitive complex (8). The density of the intermediates was determined as 1.10 to 1.13 for the detergent-resistant complex (containing mostly Pr55 and Pr160<sup>Gag-Pol</sup> precursors) and 1.15 to 1.17 for the detergent-sensitive complex, which is similar to the density of mature HIV-1 virions. Our sucrose gradient study also identified two complexes with different densities that shared similar Gag and Pol components as in the referenced study. We note that the densities of both complexes in our results are lower than those reported (8), possibly due to experimental differences.

To examine whether the complex containing both KIF-4 and Gag proteins can be further separated biochemically, we used size-exclusion and ion-exchange chromatographies. Fractions 14 to 16 from the sucrose gradient separation, which were positive for both KIF-4 and HIV-1 Gag proteins by Western blot analysis (Fig. 3), were pooled and diluted 1:1 in the running buffers for chromatography.

For separating proteins by size, we used a Bio-Prep column 1000/17 (Bio-Rad Laboratories, Hercules, Calif.) that gener-

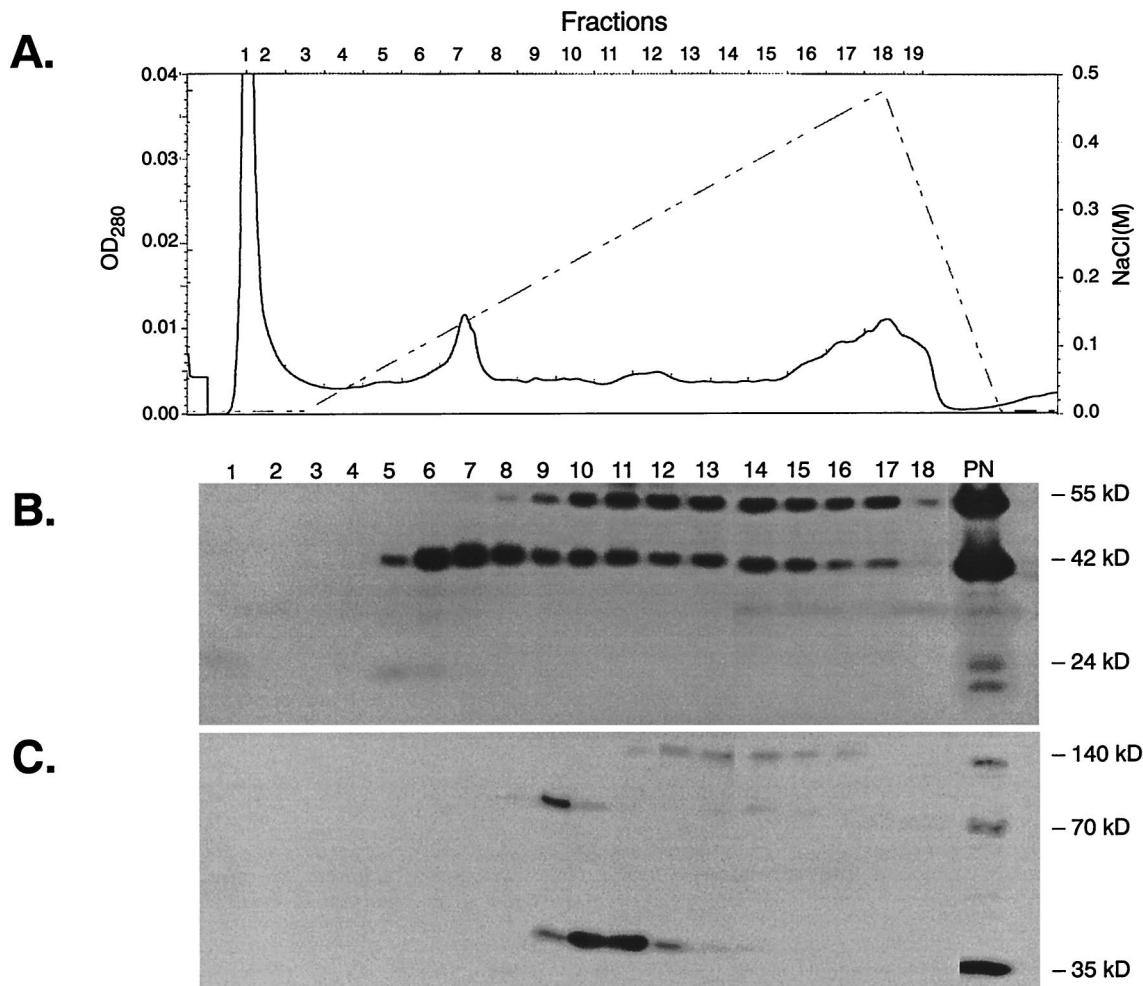


FIG. 5. Anion-exchange chromatography analysis of vVK-infected HeLa cells. Fractions 14 to 17 from sucrose gradient separation were pooled and passed through an UNO Q-1 column. (A) Histogram of protein distribution in each fraction with salt concentration (20 mM Tris or 20 mM Tris plus 1.0 M NaCl; dotted line) and conductivity (solid line) during separation; (B and C) Western blot analysis with anti-HIV-1 Pr24 Gag (B) and anti-KIF-4 (C). PN, postnuclear cell lysate; OD<sub>280</sub>, optical density at 280 nm.

ates linear fractionation from 10 to 1,000 kDa. A total of 0.5 mg of soluble protein in 0.3 ml of H-S running buffer was loaded, and the running procedure was performed under the Bio-Logic System (Bio-Rad) with a flow rate of 0.5 ml/min. Forty-one fractions of 0.5 ml were collected and pooled into eight groups according to the distribution of the peaks shown in Fig. 4A. Protein pellets obtained by ultracentrifugation with a Beckman SW50.1 rotor at 40,000 rpm for 1 h at 4°C were suspended in 1× sample buffer, boiled, and loaded onto 12% polyacrylamide-Tris-glycine gels (Novex Experimental Technology, San Diego, Calif.) for Western blotting. Figure 4B shows that the majority of HIV-1 Gag protein is present in group 5 and a small amount is present in groups 2 and 4. There is more Pr42 and less Pr55 in group 5 but more Pr55 than Pr42 in groups 2 and 4. No additional processed Gag proteins were detected in any groups. Full-length KIF-4 proteins were mainly recovered in group 5 (Fig. 4C), in which the majority of Pr42 was present. A small amount of full-length KIF-4 was also detected in group 4, where mainly Pr55 was found. Some small bands reacting with anti-KIF-4 antibody can be seen in groups 5, 7, and 8. They probably represent either processed or degraded KIF-4 molecules. A postnuclear fraction from unin-

ected HeLa cells was also examined. KIF-4 eluted one or two fractions ahead of its position in material from infected cells (data not shown).

Separation of proteins by their surface charge was accomplished with ion-exchange chromatography using a UNO Q-1 column (Bio-Rad) in accordance with the manufacturer's directions. A total of 0.5 mg of soluble protein in a total of 0.5 ml was loaded onto the column. The running buffer was started with 20 mM Tris (buffer A, pH 8.2), followed by increasing concentrations of buffer B (20 mM Tris plus 1.0 M NaCl [pH 8.2]) to elute the proteins. Eighteen fractions were collected (Fig. 5A). The majority of Gag Pr55 and Pr42 proteins were recovered in fractions 9 to 18, with a peak around fractions 10 to 15 (Fig. 5B). Substantial amounts of Pr42 and some Pr24 were eluted in fractions 6 to 9 at lower salt concentrations. Full-length KIF-4 protein was present in fractions 11 to 16 (Fig. 5C), which corresponds to the peak concentration of full-length Gag and Pr42. Smaller anti-KIF-4 antibody-reactive bands of 70 and 35 kDa were also detected in fractions 7 to 9 and 13 to 15, respectively. The 35-kDa band was consistently detected in vVK-infected cells but not in cells infected by other viruses, suggesting that a vaccinia protease may cut KIF-4 to

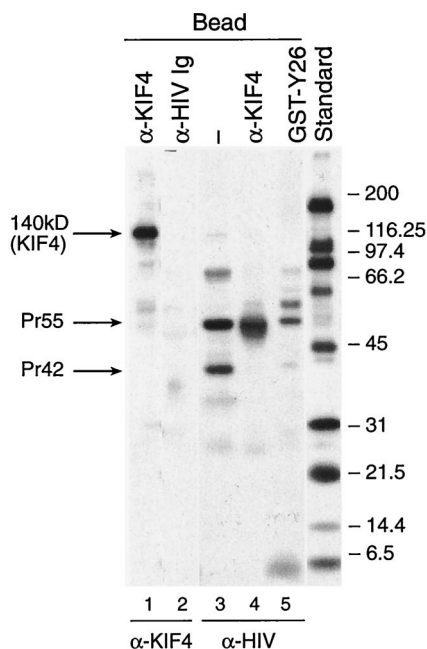


FIG. 6. Coimmunoprecipitation analyses of vVK-infected HeLa cells. Antibody-conjugated beads, anti-KIF-4 on Sepharose 4B (lanes 1 and 4) or anti-HIV-1 on protein A beads (lane 2), and GST-Y26 fusion protein on Sepharose (lane 5) were incubated with a postnuclear fraction of vVK-infected HeLa cells. Blots were developed with either anti-KIF-4 polyclonal antibody (left two lanes) or anti-HIV-1 Pr24 Gag monoclonal antibodies (right three lanes). Lane 3 is a positive control for Gag in the postnuclear fraction of vVK-infected HeLa cells.

yield a 35-kDa fragment. A very small amount of Pr24 protein was detected in fractions 4 and 5, which could be caused by a further proteolysis of Gag during separation. The full-length KIF-4 protein, however, was always associated with Pr55 and Pr42 Gag proteins. Similar results were obtained in two further studies using vVK-infected HeLa cells and in two analyses of vVK-infected BHK 21 cells. The fact that a KIF-4-HIV Gag complex in vVK-infected postnuclear supernatant remained intact during chromatographic separations following sucrose density fractionation suggests that the association between KIF-4 and Gag proteins is fairly stable. It is not known whether other cellular proteins are required for this association.

**Identification of KIF-4-HIV-1 Gag association by coimmunoprecipitation.** Immunoprecipitation studies with both anti-HIV-1 serum and anti-KIF-4-conjugated beads were used to study the KIF-4-Gag association in either vVK-infected HeLa cells or HeLa cells transfected with the pNL4-3 HIV-1 proviral clone. Briefly, cells were plated at  $10^6/60$ -mm dish and were transfected the next day with 25  $\mu$ g of proviral DNA per dish. Transfected cells were cultured for another 2 days before lysing.

Affinity-purified anti-mouse KIF-4 polyclonal antibody (11), which cross-reacts with KIF-4s in human, monkey, and hamster cells (data not shown), was conjugated to Sepharose 4B beads (6). Human HIV-1-positive serum (NIH AIDS Research and Reference Reagent Program) was conjugated to immobilized protein A-agarose beads (Pierce) at a concentration of 1 mg/ml of beads. Y26 was expressed as a glutathione *S*-transferase (GST) fusion protein and conjugated to Sepharose 4B beads as described previously (6).

After incubation of the postnuclear fraction of vVK-infected HeLa cells with anti-KIF-4 antibody-conjugated beads or GST-

Y26 fusion protein-bound beads, Pr55 Gag, but not processed Gag, was found to coprecipitate (Fig. 6, lanes 4 and 5). In contrast, no KIF-4 was detected after precipitation with anti-HIV-1 beads (Fig. 6, lane 2). Lane 3 shows the Gag proteins in the postnuclear supernatant, and lane 1 is a positive control for KIF-4 in the lysate after anti-KIF-4 bead precipitation. It has always been more difficult to bring down KIF-4 with anti-HIV-1 antibodies, either monoclonal or polyclonal, than to coprecipitate Gag proteins with anti-KIF-4 antibody by using lysates of vVK-infected or pNL4-3-transfected cells.

Our previous studies of MuLV-infected cells also showed that only a small portion of KIF-4 and Gag proteins are bound to each other (6). The results described above suggest that the beads conjugated with anti-HIV-1 or anti-Gag antibodies could primarily react with "free Gag," resulting in a much reduced chance for the KIF-4-Gag complex to be pulled down during immunoprecipitation, especially in cell lines that over-express Gag protein. On the other hand, the precipitation with anti-KIF-4 antibody has a better chance to bring down Gag proteins because the limited amount of cytoplasmic KIF-4 could allow KIF-4-Gag complex to bind to the antibody-conjugated beads.

Purified HIV-1 particles from pNL4-3-transfected or vVK-infected HeLa cells, and other sources (10), were lysed with NP-40 and incubated with beads to see whether KIF-4 is present in mature virions. No full-length KIF-4 species were found, although smaller bands that interact with anti-KIF-4 antibody could be seen by direct Western blot analysis or after immunoprecipitation (data not shown). Again, the significance of the smaller KIF-4 fragments in KIF-4-Gag association is unclear.

In summary, our studies have shown that the cellular motor protein KIF-4 associates with a number of Gag proteins, including HIV-1 Gag. This association was initially observed in the YTHS and confirmed in mammalian cells by cell fractionation, chromatography, and coimmunoprecipitation studies. KIF-4 has been described as a cellular motor protein that may transport cytoplasmic proteins or vesicles to the cell membrane. Our data now raise the possibility that KIF-4 may be involved in the transport of Gag proteins in retrovirus-infected cells.

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