

## Binding of Murine Leukemia Virus Gag Polyproteins to KIF4, a Microtubule-Based Motor Protein

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**A cDNA clone encoding a cellular protein that interacts with murine leukemia virus (MuLV) Gag proteins was isolated from a T-cell lymphoma library. The sequence of the clone is identical to the C terminus of a cellular protein, KIF4, a microtubule-associated motor protein that belongs to the kinesin superfamily. KIF4-MuLV Gag associations have been detected in vitro and in vivo in mammalian cells. We suggest that KIF4 could be involved in Gag polyprotein translocation from the cytoplasm to the cell membrane.**

The *gag* gene of murine leukemia viruses (MuLVs) encodes a Pr65<sup>Gag</sup> polyprotein that is responsible for virion particle formation, assembly, and budding at the cell membrane (reviewed in reference 26). Although N-terminal myristylation of the polyprotein has been identified as critical for plasma membrane targeting, many details of Gag protein folding, transport, membrane binding, and assembly into MuLV particles are not fully understood (11).

Previously, it was found that an immunodeficiency syndrome of mice, murine AIDS, is induced by a Pr60 variant of Gag encoded by a replication-defective virus designated BM5def (2) or Du5H (1). To explore the mechanisms by which the Pr60<sup>Gag</sup> protein of BM5def contributes to murine AIDS, we utilized the yeast two-hybrid system to screen cellular proteins that are capable of binding to this unique Gag protein. During the course of this screening, we found that the Pr60<sup>Gag</sup> protein of BM5def and the Pr65<sup>Gag</sup> protein of ecotropic MuLV bind to a cellular protein called KIF4 (21), a member of the kinesin superfamily of motor proteins (9, 12–14).

Previous studies have shown that KIF4 is a ubiquitously expressed protein especially abundant in juvenile neurons and lymphatic tissue (21). It has a microtubule plus-end-directed motor activity and is associated with small punctate structures in cultured nonneuronal cells and in neuronal growth cones (21). Thus, it was postulated that KIF4 is a motor for transport toward the cell membrane, although its specific cargo in normal cells has not yet been elucidated. Therefore, the finding of a Gag-KIF4 association suggests that KIF4 might play a role in delivering retroviral Gag polyproteins to the plasma membrane.

**Identification of proteins that interact with Pr60<sup>Gag</sup> by use of the yeast two-hybrid system.** A cDNA library prepared from the C57BL/Ka mouse V13 T-lymphoma cell line and cloned into the GAL4 activation domain expression vector pACT was purchased from Clontech (Palo Alto, Calif.). The *gag* genes of BM5def and BM5eco were amplified by Vent DNA polymerase (New England Biolabs, Beverly, Mass.) with synthetic oli-

gonucleotide primers containing *Eco*RI sites. The primers for amplification of BM5def clone 27 (2) were 5'-GATCGAATT CATGGGACAGACCATAACCAC-3' (sense) and 5'-GACT GAATTCCTAGTCACCTAAGGTTAGGA-3' (antisense). The primers for amplification of BM5eco clone 12 (2) were 5'-GATCGAATTCATGGGACAGACCAGTAACCAC-3' (sense) and 5'-GATCGAATTCCTAGTCATCTAAGGTCAGGA-3' (antisense). The amplified products were digested with *Eco*RI and cloned into the pGBT9 DNA-binding domain vector (Clontech) to generate plasmids encoding BM5def Gag (pGBT9-Pr60<sup>def-Gag</sup>) or BM5eco Gag (pGBT9-Pr65<sup>eco-Gag</sup>) fusions.

To screen the V13 T-lymphoma cDNA library, *Saccharomyces cerevisiae* HF7C cells carrying a *GAL1-lacZ* fusion gene were first cotransformed by the lithium acetate method with pGBT9-Pr60<sup>def-Gag</sup> and the lymphoma cDNA library. The transformed cells were plated out on SD synthetic medium without His, Trp, or Leu to select for cells with histidine, tryptophan, and leucine prototrophy.  $\beta$ -Galactosidase activity was assayed on nitrocellulose filter replicas of yeast transformants. Individual positive colonies were isolated, replated, and retested sequentially for  $\beta$ -galactosidase activity. Plasmid DNA was isolated from the blue colonies and used to transform *Escherichia coli* DH5 or DH10B (Life Technologies, Grand Island, N.Y.). Of 150,000 colonies screened, 31 true-positive clones were obtained and used to transform bacteria.

Minipreps of the 31 bacterial clones were digested with *Xho*I. Three 1.4-kb inserts out of 20 different-sized *Xho*I inserts were detected. One of them, which we designated Y26, was nick translated and used as a probe in Southern blots. The results showed that Y26 hybridized with itself and the other two inserts of apparently identical size (data not shown).

To determine whether the protein encoded by the Y26 clone would also react with ecotropic virus Pr65<sup>Gag</sup>, the clone was rescreened against yeast cells transformed with a construct encoding BM5eco Gag (2) as a fusion protein with the GAL4 DNA-binding domain (pGBT9-Pr65<sup>eco-Gag</sup>). Y26 reacted with both Pr60<sup>Gag</sup> and Pr65<sup>Gag</sup> (data not shown). We then selected Y26 as a candidate protein for further study.

The Y26 insert of 1,622 bp was sequenced in its entirety and gave a highly significant match with the GenBank sequence for mouse KIF4 extending from bp 2577 to the end of the published sequence (21) at 3976 (data not shown; KIF4 GenBank

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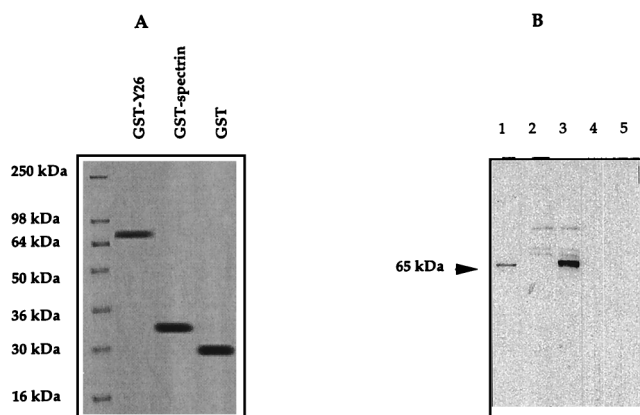


FIG. 1. Interaction between GST-Y26 protein and ecotropic virus Gag. (A) Protein conjugated to glutathione beads. Ten microliters of GST-, GST-spectrin-, and GST-Y26-conjugated beads was washed, resuspended in SDS-sample buffer, heated 10 min, and loaded on a Tris-glycine 12% polyacrylamide gel. Coomassie blue was used for gel staining. (B) Analysis of GST-Y26 protein-Gag association in vitro. Twenty microliters (each) of GST-Y26-, GST-, and GST-spectrin-bound beads was incubated with 200  $\mu$ l of cell lysates for 2 h at 4 C. Precipitates were washed and loaded on a Tris-glycine 12% polyacrylamide gel. The blot was developed with the R187 MAb. Lanes: 1, Y26 beads plus BHK-VV-eco; 2, Y26 beads plus BHK-VV; 3, 10  $\mu$ l of BHK-VV-eco lysate only; 4, GST beads plus BHK-VV-eco; 5, GST-spectrin beads plus BHK-VV-eco.

accession no. D12646). In the single large open reading frame (ORF) common to both sequences, only 2 bp distinguished the Y26 sequence from the published sequence for KIF4: an A for a G at 3254 and a G for an A at 3544 of the KIF4 sequence. Although the Y26 cDNA expressing the Gag-binding protein did not contain the full-length *KIF4* transcript, the sequence of the captured insert and the published sequence of the carboxy terminus of the full protein were essentially identical. It is unlikely that the differences seen indicate that the captured cDNA derives from kinesins other than KIF4, because other members of this protein family show the most divergence from one another in the carboxy terminus (9, 12–14). We conclude that Y26 encompasses the carboxy terminus of KIF4.

**Y26 binds to Gag polyproteins in vitro.** A GST-Y26 fusion was constructed by subcloning the 1.5-kbp *Xho*I insert of Y26 into the pGEX-4T-2 vector (Pharmacia Biotechnology, Piscataway, N.J.). The resulting construct, pGEX-4T-2-Y26, was grown in *E. coli* BL21. GST-Y26 fusion protein was induced for 6 h from BL21/pGEX-4T-2-Y26 with IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside) and purified by affinity binding to glutathione-Sepharose 4B according to the procedures recommended by the manufacturer (Pharmacia Biotechnology). The GST-Y26 concentration on the beads was 2  $\mu$ g/ml. The pGEX-4T-2 vector and pGEX-spectrin (kindly provided by David Bowtell, Peter MacCallum Cancer Institute, Melbourne Australia) were also grown in *E. coli* BL21 to generate glutathione S-transferase (GST) protein and GST-spectrin fusion protein for negative controls. The concentrations of those proteins on beads were 4  $\mu$ g/ml (Fig. 1A).

Recombinant vaccinia virus strains expressing the BM5 defective virus *gag* gene or the BM5 ecotropic virus *gag* gene were constructed. For the defective *gag* gene, a 1.75-kb *gag* ORF plus 120 bp of 5' noncoding sequences isolated from plasmid pBM5DEF27 (2) was ligated with a pTK-gpt-selP vector derivative (6, 7, 18). For the ecotropic *gag* gene, a 1.8-kb *Ban*II fragment containing the *gag* ORF from the plasmid pBM5ECO 12-1 (2) was ligated with a *Ban*II-cleaved pTZ-L2 vector derivative (18). CV-1 cells previously infected with wild-

type vaccinia virus were transfected with plasmid DNAs to generate recombinant vaccinia viruses.

BHK21 cells were cultured in Dulbecco's modified Eagle's medium (Quality Biological, Inc., Gaithersburg, Md.) containing 5% fetal calf serum. Cells were infected with wild-type vaccinia virus or vaccinia virus recombinants expressing either BM5def or BM5eco Gag at a virus concentration of 0.05 PFU/cell for 24 to 48 h. Cells were then lysed in Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.0), 0.5% Nonidet P-40, 5 mM EDTA, 0.5% Tween 20, 10  $\mu$ g of aprotinin and leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride) at a concentration of  $2 \times 10^7$  cells/ml. Cell lysates were pre-cleared by overnight incubation at 4°C with rat or rabbit immunoglobulin G (IgG)-coupled Sepharose beads. Total protein concentrations were measured by the Bradford method (Bio-Rad Laboratories, Hercules, Calif.).

GST- or GST-Y26 fusion protein-conjugated beads were incubated with pre-cleared cell lysates at a ratio of 1:10 to 1:20 (vol/vol) for 2 h at 4°C. The beads were then washed twice in buffers containing 600, 300, and 150 mM NaCl and 1 mM phenylmethylsulfonyl fluoride individually, resuspended in 40  $\mu$ l of sodium dodecyl sulfate (SDS) reducing sample buffer, boiled for 5 min, and loaded on Tris-glycine 12% polyacrylamide minigels (Novex Experimental Technology, San Diego, Calif.). Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. For the Western blot procedure, the manufacturer's protocol was followed (version L.4; Tropix, Bedford, Mass.).

The lysate of BHK21 cells infected with a vaccinia virus recombinant expressing the BM5 ecotropic *gag* gene (BHK-VV-eco) was used as a source of Gag polyproteins and as a positive control (Fig. 1B, lane 3). Pr65<sup>Gag</sup> was detected following incubation of GST-Y26 beads with lysates of the BHK-VV-eco lysate (Fig. 1B, lane 1). No Gag polyproteins were detected following incubation of GST-Y26 beads with lysates of cells infected with wild-type vaccinia virus (BHK-VV) (lane 2) or incubation of the BHK-VV-eco lysate with beads conjugated with GST protein alone or the GST-spectrin fusion protein (lanes 4 and 5).

**KIF4 binds to Gag polyproteins in vivo.** Polyclonal rabbit anti-KIF4 antibody (21) and anti-Gag p12 monoclonal antibody (MAb), 548, obtained from Bruce Chesebro (National Institute of Allergy and Infectious Diseases) (5), were covalently linked to Sepharose beads by established techniques (25) at a concentration of 1 mg/ml. Nonimmune rabbit IgG or a rat MAb to a B-cell surface antigen, BRB44, was also conjugated to Sepharose beads at the same concentration and used as a negative control. Immunoprecipitations with beads were performed with the lysates of BHK-VV-eco or BHK-VV as described above. The amount of lysate protein used in each reaction was about 200  $\mu$ g. The blots were probed with anti-Gag p30 MAb (R187) (Fig. 2A) or polyclonal antibody to KIF4 (Fig. 2B).

The 65-kDa Gag protein was detected in anti-KIF4 precipitates with the lysate of BHK-VV-eco (Fig. 2A, lane 4), but not in negative control precipitates (lanes 2 and 3). Conversely, the KIF4 protein with a size of approximately 140 kDa was detected in the anti-p12 precipitates after incubation with the same lysate (Fig. 2B, lane 4), but not in control samples (lanes 2 and 3). Normal expression of Pr65<sup>Gag</sup> and KIF4 was seen in lanes 1 of Fig. 2A and B, respectively. These results demonstrate that native KIF4 and Gag of BM5 ecotropic virus associate in vivo when Gag is overexpressed from a vaccinia virus vector.

We next asked whether the KIF4-Gag association can be detected in normal retrovirus-infected cells. To answer this

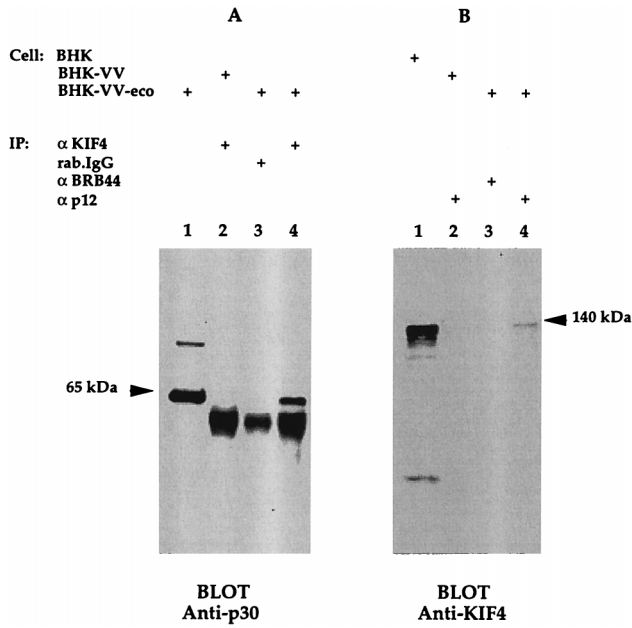


FIG. 2. KIF4-Gag association in BHK cells. Lysates were prepared from BHK21 cells either uninfected (BHK) or infected with wild-type vaccinia virus (BHK-VV) or vaccinia virus expressing ecotropic MuLV Gag (BHK-VV-eco). Two hundred microliters of lysates containing approximately 200 μg of cytoplasm protein was precipitated with 10 μl of Sepharose beads conjugated with polyclonal rabbit IgG anti-KIF4 (KIF4), with normal rabbit IgG (rab.IgG), with rat MAb anti-BRB44 (BRB44), or with rat anti-Gag p12 MAb (p12). Precipitated proteins were separated on Tris-glycine 10% polyacrylamide gels and transferred to PVDF membranes before being blotted with the indicated antibodies. IP, immunoprecipitation.

question, SC-1 (10) and *Mus dunni* (15) cells were infected with pools of viruses as described previously (20). The SC-1 cells were infected with ecotropic Moloney virus (22) or amphotropic 4070A virus (4), while *M. dunni* cells were infected with AKR 13 mink cytopathic focus-forming (MCF) virus (3) or NZB IU6 xenotropic virus (3). Infected cells were harvested for biochemical analysis on day 5 after infection. Cells were lysed and immunoprecipitated as described above. The Gag and KIF4 proteins were detected by anti-KIF4 antibody or MAb R187 under the same conditions as those described above (Fig. 3). Pr65<sup>Gag</sup> polyproteins were detected in lysates of

Moloney virus and 4070A virus infected SC-1 cells precipitated with anti-KIF4 beads (Fig. 3 [left], lanes 2 and 3) and were also in AKR 13-infected *M. dunni* cells (lane 5), but not in NZB IU6-infected *M. dunni* cells (lane 6). In precipitations with R187 beads, the 140-kDa band of KIF4 was detected in ecotropic, amphotropic, and MCF virus-infected SC-1 or *M. dunni* cells (Fig. 3 [right], lanes 2, 3, and 5), but not in xenotropic virus-infected cells (lane 6). The in vivo coimmunoprecipitation results indicate that the KIF4-Gag association can be detected in most retrovirus-infected cells tested in this experiment.

**KIF4 expression in mouse tissues.** If the observed KIF4-Gag association in retrovirus-infected cells is relevant to normal retrovirus biology, then KIF4 should be expressed ubiquitously, because retrovirus expression can be detected in virtually all tissues of mice that express endogenous ecotropic virus at high levels (19). We therefore examined the expression of KIF4 in multiple mouse tissues by using both reverse transcription-PCR and immunoblot analyses. Because KIF4 is known to be expressed at high levels in lymphocytes, we studied tissues from a Rag2 knockout mouse (Taconic, Germantown, N.Y.) incapable of producing mature lymphocytes to ensure that signals for KIF4 in lysates of different tissues did not represent infiltrating T and B cells.

Total RNA was isolated from mouse tissues with the use of RNazol B (Tel-Test, Inc., Friendswood, Tex.) by the protocol of Svetic et al. (23). cDNA synthesis, PCR, Southern blotting, and chemiluminescent detection were performed as described by Gazzinelli et al. (8). For the PCRs, primers were synthesized corresponding to the KIF4 sequence for bases 3766 to 3788 (5'-TCTTCCAGTCTCCAGACTCTTCC-3') and the complement to bases 4396 to 4418 (5'-CTCTTCTTGGATAGGAGAGCAGC-3'). PCR conditions were 95, then 60, and then 72°C for 60 s each for 30 cycles, except (i) the first cycle was 180 s at 95°C, and (ii) the last cycle was 300 s at 72°C. The magnesium chloride concentration was 1.25 mM. The number of cycles chosen for blot analysis was well short of saturation. The probe used for KIF4 detection by hybridization and chemiluminescence was 5'-GCCGAGCAGGACAATGAG-3' (KIF4 bases 3894 to 3911) (21).

Multiple tissue samples (40 to 50 mg) from a Rag2 gene-knockout mouse and a spleen from a wild-type C57BL/6 mouse were homogenized and lysed in 1 ml of Nonidet P-40 lysis buffer. Postnuclear lysates were separated on Tris-glycine 12%

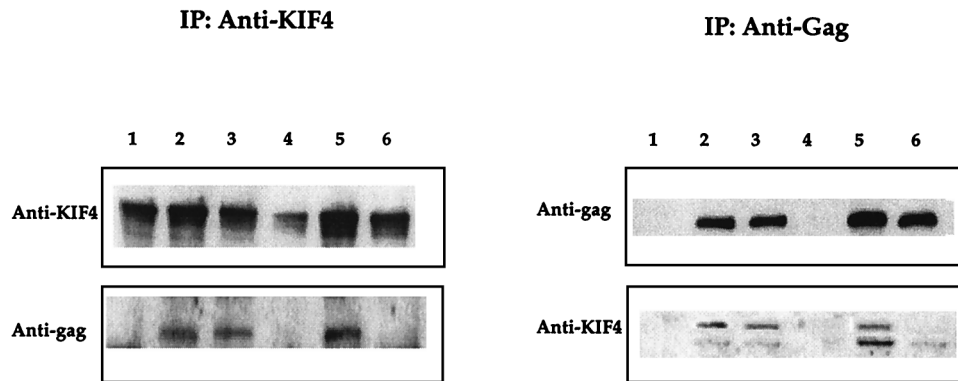


FIG. 3. KIF4 associates with Gag in multiple MuLV-infected cells. Lysates were prepared from uninfected SC-1 cells (lane 1) and SC-1 cells infected with ecotropic (lane 2) or amphotropic (lane 3) viruses and from uninfected *M. dunni* cells (lane 4) and *M. dunni* cells infected with MCF (lane 5) or xenotropic (lane 6) MuLV. Equivalent amounts of proteins and beads were used as described in the legend to Fig. 2. Precipitated proteins were separated on a Tris-glycine 12% polyacrylamide gel and transferred to PVDF membranes before being blotted with the indicated antibodies. IP, immunoprecipitation.

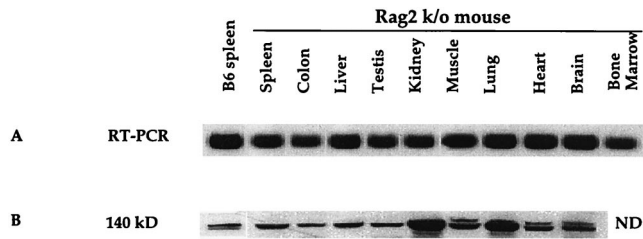


FIG. 4. KIF4 expression in mouse tissues. (A) RT-PCR of total RNA from the indicated tissues of a Rag2 knockout (k/o) mouse and spleen tissue from a normal C57BL/6 (B6) mouse. (B) KIF4 protein expression in the tissues described above. Lysates from each tissue were loaded on a Tris-glycine 12% polyacrylamide gel, transferred to a PVDF membrane, and then developed with anti-KIF4 antibody. ND, not done.

polyacrylamide minigels (Novex Experimental Technology). The blot was probed by anti-KIF4 antibody (21).

All tissues tested were positive both by RT-PCR (Fig. 4A) and by immunoblotting (Fig. 4B). Thus, KIF4 is widely expressed and would be available to participate in virus particle formation in a variety of tissues. This result is apparently different from that of a previous study, which had shown that KIF4 transcripts were not detectable by Northern blotting in adult mouse tissues other than spleen (21). The different techniques used for the analysis of KIF4 expression may be responsible for this difference. The results reported in our study reinforce the previous result that KIF4 is ubiquitously expressed in almost all mouse tissues, although its expression level decreases during development. This conclusion is further supported by KIF4 expression in human (HeLa), monkey (Cos-7), and hamster (BHK21) cells, although expression levels were variable (17, 24).

Our studies have shown that only a small amount of KIF4 can be coimmunoprecipitated by anti-Gag antibodies and that only low levels of Gag are seen in the anti-KIF4 precipitates. This may suggest that only a small part of cellular KIF4 is recruited for the transport of Gag and/or that KIF4 rapidly dissociates from Gag when it reaches the plasma membrane (16). To clarify these issues, biochemical characterization of the interaction of purified KIF4 and Gag protein, as well as its regulation, will be important. Studies are in progress to determine if KIF4 binds to the Gag polyproteins of other members of the retrovirus family. The subcellular localization and the molecular basis for this protein-protein interaction are also under investigation.

The first and second authors contributed equally to this research.

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