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Identification of a Cellular Protein Interacting with Murine Retrovirus Gag Polyproteins

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The retroviral Gag polyprotein directs the assembly of virion particles and plays an important role in some events after entry into a host cell. The Gag polyprotein of a virus mixture is responsible for inducing murine acquired immunodeficiency syndrome (MAIDS) when injected into susceptible strains of mice. In order to identify the host cellular proteins which interact with the MAIDS virus Gag proteins and possibly mediate the function of the Gag proteins, mouse T-cell leukemic cDNA expression library was screened using the yeast GAL4 two hybrid system. Of 11 individual positive clones, the clone Y1 was selected for the study of protein-protein interaction. Its DNA sequence revealed that it was an exact match to the murine SH3 domain-containing protein SH3P8. It is expressed as 2.4 kbp transcripts in testis at higher levels and in various tissues tested at lower levels. Glutathione S-transferase-Y1 fusion protein binds tightly to $Pr60^{def\text{-}gag}$ as well as $Pr65^{eco\text{-}gag}$.

Key words: Gag polyproteins, yeast two hybrid system, protein-protein interactions, SH3 domain-containing protein, GST-fusion protein

The gag gene product of murine leukemia virus (MuLV), termed $Pr65^{gag}$, mediates virion assembly (1, 2). It is synthesized by free ribosomes as a precursor polyprotein. During or shortly after budding, gag precursor polyprotein is cleaved by the viral protease into four peptide components: matrix (MA or p15), which is in close association with the viral membrane surface, p12 of unknown function, capsid (CA or p30), which forms the core of the virion, and nucleocapsid (NC or p10), which coats the genomic RNAs.

A mixture of C-type murine leukemia viruses (MuLV) induces murine AIDS (MAIDS) after infection of susceptible strains of mice (3, 4). This virus mixture, designated LP-BM5, consists of non-pathogenic, replication-competent, B-tropic ecotropic MuLV (referred to as BM 5eco), mink-cell focus forming (MCF) MuLV and a disease-inducing defective genome (referred to as BM 5def). BM5def genome encodes only functional Gag precursor protein of 60 kd ($Pr60^{def\text{-}gag}$)(5, 6). Deduced amino acid sequence of the gag genes of BM5def (def-gag) and BM5eco (eco-gag) indicates that carboxy-terminal half of MA and much of p12 vary considerably between the two gag genes including 15-bp deletion in the p12 region of def-gag, whereas only six different amino acids are scattered over the CA and NC region (4).

The mechanisms by which $Pr60^{def\text{-}gag}$ contribute to the pathogenesis of MAIDS are not well understood, but two models have been suggested. The "superantigen" model was derived from the observation that T-cell subsets respond massively to $Pr60^{def\text{-}gag}$ (7). The "transforming activity" was hypothesized to be responsible for frequent occurrence of lymphomas in infected mice with LP-BM5 mixture (3). For better understanding the mechanisms of MAIDS pathogenesis, biochemical characteristics, if any, of $Pr60^{def\text{-}gag}$ should be clarified. Yeast two-hybrid system was utilized in search of a host cellular protein(s) which interacts with $Pr60^{def\text{-}gag}$ and possibly contributes to the pathogenesis of MAIDS.

The yeast two hybrid system is a method for studying protein-protein interactions (8). Yeasts with two reporter genes, LacZ and His3, under the control of the GAL4 promoter are cotransformed with two plasmids encoding a fusion protein between GAL4 DNA-binding domain and protein X, and a fusion protein between GAL4 activation domain and protein Y. If protein X interacts with protein Y inside the nucleus of yeast, the two domains of GAL4 are located closely enough to reconstitute transactivation function, which leads to the ex-

pression of His3 reporter gene. Primary His+ transformants are tested for expression of the second easily assayable reporter gene, LacZ, to reduce the background of false positives arising in the His selection.

A mouse library encoding proteins which interact with the Gag polyproteins of mouse AIDS virus has been screened. One clone, Y1, was selected for further analyses, because it was represented most frequently among the positive clones; and it also showed a rapid color reaction indicating strong interaction with the MAIDS Gag polyproteins.

Materials and Methods

Recombinant constructs

The gag genes of BM5def and BM5eco were amplified by Vent polymerase (New England Biolabs) using synthetic oligonucleotides containing EcoRI linkers. The primers for amplification of BM5def clone27 were 5'-GATCGAATTCATGGGACAGACCATAACCAC-3' (sense) and 5'-GATCGAATTCCTAGTCACCTAAGGTTAGGA-3' (anti-sense). The primers for amplification of BM5eco clone12 were 5'-GATCGAATTCATGGGACAGACCGTAACCAC-3' (sense) and 5'-GATCGAATTCCTAGTCATCTAAGGTGAGGA-3' (anti-sense). The amplified products were digested with EcoRI and cloned into the pGBT9 DNA binding domain vector (Clontech) which contained Leu2 marker to generate vectors containing BM5def-gag or BM5eco-gag (pGBT9-Pr60$^{def\text{-}gag}$ or pGBT9-Pr65$^{eco\text{-}gag}$).

cDNA library screening

A cDNA library prepared from the C57 BL/Ka V13 T cell lymphoma line was purchased from Clontech and cloned into the XhoI site of pACT activation domain vector which contains Trp1 marker. *S. cerevisiae* HF7C (Clontech), which harbors His3 and LacZ as reporters, was cotransformed sequentially with pGBT9-Pr60$^{def\text{-}gag}$ and the lymphoma cDNA library DNAs using the lithium-acetate method (9). The transformed cells were plated on SD synthetic medium which consists of a yeast nitrogen base, 2% dextrose, and a stock of dropout solution without histidine, leucine, and tryptophan to select cells with histidine, leucine, tryptophan prototropy.

β-gal assay was performed on Whatmann filter replicas of yeast transformants (10). Individual blue colonies were retested for β-gal activity. Plasmid DNA was isolated from the positive clones and used to transform *E. coli* DH5 or DH10B for DNA sequencing (11).

Sequence analysis

DNA sequence of Y1 cDNA insert was obtained by dideoxy sequencing using oligonucleotide primers from pACT (5'-TACCACTACAATGGATG-3' and 5'-CAGTTGGAAGTGAACTTGC-3') and several internal primers.

Northern analysis

A mouse multiple-tissue Northern blot was purchased from Clontech. Y1 insert DNA was excised by XhoI digestion and ^{32}P-labeled by nick translation. After a moderately stringent wash (0.1X SSC, 0.1% SDS at 55°C), the blot was exposed to X-ray film at -70°C overnight with two intensifying screens.

Preparation of GST-Y1 fusion protein

A GST-Y1 fusion vector was constructed by subcloning 1.7 kbp XhoI insert into pGEX-4T-2 (Pharmacia Biotechnology). The resulting vector, pGEX4T-2-Y1, was used for transformation of *E. coli* strain BL21. GST-Y1 fusion protein was prepared according to the procedures recommended by the manufacturer (12). Transformed BL21 was cultured to an A_{600} of 0.6-0.8 with vigorous agitation at 37°C. Proteins were induced by adding IPTG to a final concentration of 1 mM and continued incubation for additional 1-2 hours. Bacteria were pelleted and washed in ice-cold PBS. The suspended bacteria in ice-cold PBS at a ratio of 20:1 were sonicated on ice in short bursts until a clear lysate was obtained. Bacterial sonicates were treated with Triton X-100 to a final concentration of 1% to aid solubilization of the fusion protein. Insoluble material was pelleted at 12,000×g for 10 min at 4°C. A 50% slurry of Glutathione Sepharose 4B (Pharmacia) equilibrated with 1 X PBS was added to the supernatant at a 50:1 ratio. After incubation with gentle agitation at room temperature, the fusion protein/Sepharose 4B bead matrix was centrifuged. The supernatant was removed, and the pellet was washed three times with 10 bead volumes of PBS. The fusion protein was eluted from the matrix by adding glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) with the same ratio of buffer to bead volume of matrix. After incubation at room tepmperature for 10 min, the protein/bead mixture was centrifuged, and the fusion protein were saved from the supernatant.

In vitro binding assay

Binding assay was performed as described previously (13). G beads (Pharmacia) were swollen overnight at 4°C in TEK buffer [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM KCl], washed three times in TEK buffer with 0.5% powered milk, and stored at 4°C in binding buffer [20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.5 mM phenylmethlsulfonyl fluoride, and 5% glycerol]. Typical binding

reactions used crude lysates of baby hamster kidney cell line expressing $Pr60^{def\text{-}gag}$ or $Pr65^{ecr\text{-}gag}$ in a total volume of 200 μl binding buffer containing 5 g of GST-Y1 (approximately 0.5 M). After incubation at 4℃ on a rotator, 25 ml of 50% (v/v) G beads in binding buffer was added, and incubation was continued for another 30 min. The G beads were collected with a 5 s pulse in a microfuge and washed three times with 400 μl of binding buffer. Washed G beads were resuspended in 25 μl of 2×SDS sample buffer, heated in boiling water for 5 min, and pelleted in a microfuge. The supernatant (5 μl) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were either stained with Coomassie blue or processed for Western blot analysis

Western blot analysis

Proteins were electrotransferred to nitrocellulose membrane with the Boi-Rad mini-blotting apparatus. The blot was incubated with the mouse monoclonal anti-Gag (anti-p30) antibodies (14), followed by horse raddish peroxidase-linked rabbit anti-mouse immunoglobulin. Antibody binding was detected with ECL Western blotting detection reagents (Amersham).

Results

Identification of a protein which interacts with Pr $60^{def\text{-}gag}$

To identify host cellular proteins that interact specifically with gag polyprotein of BM5def, yeast two-hybrid system was exploited . The mouse T-cell cDNA library was created by Clontech by cloning cDNA inserts into the XhoI site of GAL4 activation domain vector, pACT. Library DNAs were used to transform yeast containing pGBT9-$Pr60^{def\text{-}gag}$ encoding a GAL4 DNA-binding domain-$Pr60^{def\text{-}gag}$ fusion protein. Of 150,000 colonies screened, 31 positive clones were identified. The plasmids from positive clones were used to transform bacteria. Minipreps of the 31 bacterial clones were used for XhoI digestion and Southern blotting. The Y1 insert of 1.7 kbp was nick-translated and used to probe Southern blot of inserts from all positive clones. Of 31 positive clones analyzed, 10 clones were found to hybridize to the labeled Y1 insert. DNA sequencing of Y1 cDNA insert revealed that it was a perfect match to the carboxy-terminal 210 amino acids of SH3-containing protein SH3P8 (Fig.1)(15-17) and showed a significant homology to two SH3P8-related proteins, SH3P4 and SH3P13 (data not shown). The SH3P8 gene is expressed as 2018 bp transcripts encoding 368 amino acids. It contains 117 bp and 1107 bp of 5'- and 3'-untranslated regions, respectively. The Y1 insert is composed of 630 bp coding region and 1107 bp 3'-untranslated region of SH3P8.

Northern analysis of Y1 transcripts

The Y1 insert was excised out with XhoI from the pACT-Y1, labeled, and used as a probe for hybridization of Northern blot of mRNA prepared from various adult mouse tissues. The Y1 probe hybridized to 2.4 kbp tran-

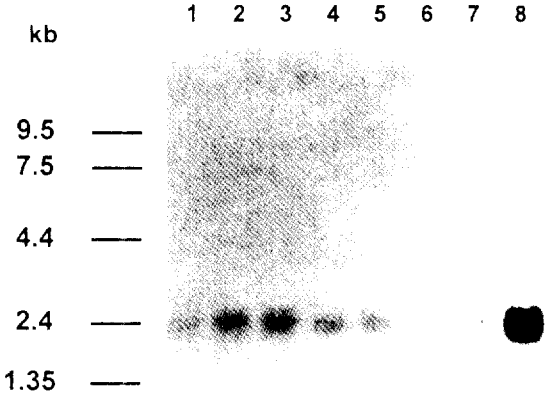

Fig. 2. Analyses of SH3P8 transcripts in tissues of adult mice. Hybridization of mouse multiple tissue Northern blot was performed with nick-translated Y1 insert as a probe. Lane 1: heart. Lane 2: brain. Lane 3: spleen. Lane 4: lung. Lane 5: liver. Lane 6: skeletal muscle. Lane 7: kidney. Lane 8: testis.

1 KKLEGRRLDFDYKKKRQGKIPDEELRQALEKFEESKEVAE

41 TSMHNLLETDIEQVSQLSALVDAQLDYHRQAVQILEELAD

81 KLKRRVREASSRPKREFKPRPREPFELGELEQPNGGFPCA

121 PAPKITASSSFRSSDKPIRMPSKSMPPLDQPSCKALYDFEP

161 ENDGELGFREGDLITLTNQIDENWYEGMLHGQSGFFPLS

201 YVQVLVPLPQ

Fig. 1. Deduced amino acid sequence of the Y1 cDNA insert. The SH3 domain is underlined. Positions corresponding to the conserved residues shown to be involved in ligand binding in the SH3 domains of Src and Grb2/Sem5 are presented in bold and dot formats, respectively.

scripts were expressed at highest levels in testis and at lower levels in various tissues (Fig. 2).

Binding of Y1 protein product to gag polyproteins in vitro

To demonstrate in vitro binding of Gag polyproteins to Y1 protein, the insert from clone Y1 was first subcloned in frame of a bacterial plasmid, pGEX 4T-2, to express Y1 protein as glutathione S-transferase (GST) fusion protein. A significant fraction of the total bacterial protein in crude lysate was constituted by GST-Y1 protein of 50 kD (data not shown). GST-Y1 fusion protein was purified in a single step using glutathione-Sepharose beads (G beads), and the purity of the protein was assessed by electrophoresis after elution with glutathione. More than 80 % of the protein had the expected mobility for the Y1 fusion protein. To demonstrate protein-protein interaction in vitro, fusion protein-bound G beads was directly used.

As a source of $Pr60^{def\text{-}gag}$ and $Pr65^{eco\text{-}gag}$, a preparation of total soluble protein from BHK21 transfected with vaccinia recombinant virus expressing $Pr60^{def\text{-}gag}$ and $Pr65^{eco\text{-}gag}$ was used. When the G beads bound GST-Y1 fusion protein were incubated with vaccinia lysates, and probed with mouse anti p30 and peroxidase-linked rabbit anti-mouse immonoglobulin, Gag polyproteins were easily detected on Western blot (Figure 3, lanes 3 and 5) or on Coomassie blue-stained gels (data not shown), whereas no Gag proteins were bound in the lysate infected with wild type vaccinia virus (Figure 3, lane 1). In addition to intact Gag polyproteins, one large product of 150 kD was reproducibly seen on a Western blot (Figure 3, lanes 2 and 4), the origin of which is not known.

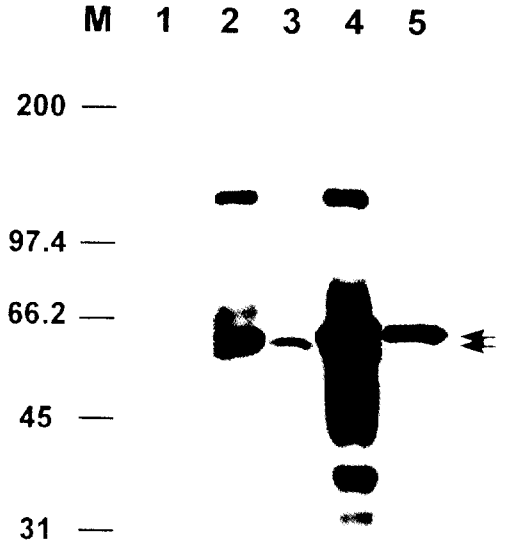

Fig. 3. Binding of Gag to GST-Y1 fusion protein. BHK21 cells were infected with wild type vaccinia virus or recombinant vaccinia virus expressing BM5def-gag or BM5eco-gag, lysed. Lane 1: BHK lysate. Lane 2: BHKdef total lysate. Lane 3: BHKdef lysate precipitated with GST-Y1 beads. Lane 4: BHKeco total lysate. Lane 5: BHKeco lysate precipitated with GST -Y1 beads. The positions of size markers are indicated in kilodalton on the left side. The top and bottom arrows on the right side represent $Pr65^{eco\text{-}gag}$ and $Pr60^{def\text{-}gag}$, respectively.

Discussion

Screening with the two hybrid system for cDNAs encoding proteins that interact with $Pr60^{def\text{-}gag}$ resulted in isolation of 31 positive clones from 1.5×10^5 colonies screened. Of these 31 clones, ten individual clones showed significant homology to Y1 insert on Southern analysis. Repeated isolation of the same clone decreased the possibility to a large extent that the interaction between gag polyproteins and SH3P8 may be an artifact. In fact this type of interaction was expected on the following reasons: SH3 domains mediate protein-protein interaction by binding proline-rich region in the target proteins; a proline-rich region exists in Gag polyproteins. Combined region of carboxy-half of p15 and the entire p 12 harbors proline residues in portion of 25 % and 23 % for $Pr60^{def\text{-}gag}$ and $Pr65^{eco\text{-}gag}$, respectively (5). To prove further the binding of SH3 domain of SH3P8 protein through the proline-rich region of Gag polyproteins, precise binding regions between the two proteins should be determined, supported by a competetion experiment and an in vivo binding experiment such as coimmunoprecipitation.

SH3 domain containing proteins are known to be involved in signal transduction (18, 19). Whether a novel SH3P8 protein is a component in that pathway is yet to be clarified. It is unlikely that the interaction of Gag protein with SH3P8 leads to the pathogenesis of MAIDS, because Gag polyproteins of both defective (BM5def) and non-pathogenic (BM5eco) viruses bound to SH3P8 protein. Rather, the interaction of Gag polyproteins with SH 3P8 protein seems to be an early event for the viral replication. The conclusion of this study, however, can not exclude the possibility that $Pr60^{def\text{-}gag}$ differs from $Pr65^{eco\text{-}gag}$ in inducing through the signal transduction pathway the expression of some genes which are necessary for MAIDS pathogenesis. Functional analyses of the remaining isolated clones could discriminate roles of $Pr60^{def\text{-}gag}$ and $Pr65^{eco\text{-}gag}$ in the induction of MAIDS.

The two models suggesting the role of $Pr60^{def\text{-}gag}$, the superantigen and the transforming activity, have never been proved yet. When $Pr60^{def\text{-}gag}$ was expressed in B-cell lines without endogenous superantigen such as MMTV orf gene, no T-cell response was observed (20). On the

other hand the transforming activity of $Pr60^{def\text{-}gag}$ has not been reproduced since the first study was published. A compromise for these two models would be reached by investigating the role(s) of $Pr60^{def\text{-}gag}$ from different points of view, i. e., as a transcription factor which acts in the nucleus for direct induction of endogenous superantigen or transforming gene. This hypothesis is contradictory to the observation that $Pr60^{def\text{-}gag\ is}$ localized in the cytosol anchored to the inner membrane through the myristilation of the second residue, glycine (21). But a very small portion of $Pr60^{def\text{-}gag}$ may migrate to the nucleus, which could not be detected by conventional immunohistological staining. The introduction of an advanced technology such as confocal microscopy could precisely localize small amount of specific proteins.

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