FUNCTIONAL DISRUPTION OF THE MOLONEY MURINE LEUKEMIA VIRUS PREINTEGRATION COMPLEX BY VACCinia-RELATED KinASES

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Running head: Dysfunction of retroviral integration complex by cellular kinases

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Retroviral integration is executed by the preintegration complex (PIC) that contains viral DNA together with a number of proteins. Barrier-to-autointegration factor (BAF), a cellular component of Moloney murine leukemia virus (MoMLV) PICs, has been demonstrated to protect viral DNA from autointegration and stimulate the intermolecular integration activity of the PIC by its DNA-binding activity. Recent studies reveal that the functions of BAF are regulated by phosphorylation via a family of cellular serine/threonine kinases called vaccinia-related kinases (VRK), and VRK-mediated phosphorylation causes a loss of the DNA-binding activity of BAF. These results raise the possibility that BAF-phosphorylation may influence the integration activities of the PIC through removal of BAF from viral DNA. In the present study, we report that VRK1 was able to abolish the intermolecular integration activity of MoMLV PICs in vitro. This was accompanied by an enhancement of autointegration activity and dissociation of BAF from the PICs. In addition, in vitro phosphorylation of BAF by VRK1 abrogated the activity of BAF in PIC function. Amongst the VRK family members, VRK1 as well as VRK2, which catalyze hyperphosphorylation of BAF, could abolish PIC function. We also found that treatment of PICs with certain nucleotides such as ATP resulted in the inhibition of the intermolecular integration activity of PICs through the dissociation of BAF. More important, the ATP-induced disruption was not observed with the PICs from VRK1-knockdown cells. Our in vitro results therefore suggest the presence of cellular kinases including VRKs that can inactivate the retroviral integration complex via BAF phosphorylation.

The core of an infecting retroviral virion is comprised of a nucleoprotein complex in which genomic RNA is associated with enzymes and many proteins that are required for completing the early phase of infection. After penetration of the cell membrane, reverse transcription of viral RNA takes place in the reverse transcription complex (RTC), a virion-derived nucleoprotein complex. The synthesis of full-length viral DNA in the RTC produces a high-order nucleoprotein complex called the preintegration complex (PIC) that subsequently executes viral DNA integration into chromosomal DNA in the infected cell (1). The PIC exhibits the full fidelity of the integration reaction that is defined as 3’-end processing and strand transfer steps (2, 3). In addition, the PICs isolated from retrovirus-infected cells efficiently insert both viral DNA ends into target DNA in a pairwise manner in vitro, whereas the simple reaction catalyzed by purified integrase mostly results in integration of a single viral DNA end into one strand of the target DNA (4-6).

A striking feature of PICs is their strong preference for integration into target DNA (intermolecular integration) and avoidance of intramolecular integration into the viral DNA itself, a reaction termed autointegration (2, 5, 7). Barrier-to-autointegration factor (BAF) has been reported as a cellular component of the PIC derived from Moloney murine leukemia virus (MoMLV) and human immunodeficiency virus type 1 (HIV-1)-infected cells, which stimulates the intermolecular integration reaction of PIC and blocks autointegration of MoMLV PIC in vitro (8-11). BAF is an 89 amino acid protein that is
highly conserved amongst metazoans (12). The functional unit of BAF is a homodimer in which each monomer binds to double-stranded (ds) DNA nonspecifically via contacts between the helix-hairpin-helix motif of BAF and phosphate backbone of DNA (8, 13, 14). On the basis of this structure, it has been proposed that DNA-bridging caused by the BAF-DNA interaction compacts viral DNA into a rigid structure within the PIC, making it inaccessible as a target for autointegration (8, 10, 14). In addition, the DNA-bridging property has been suggested to recruit PICs to target DNA, promoting intermolecular integration \textit{in vitro} (10). However, despite the insight provided by functional \textit{in vitro} data, the importance of BAF for retroviral PIC function is controversial (15, 16).

The physiological function of BAF in a cell is to regulate higher-order chromatin organization including nuclear envelope assembly and gene regulation in a cell-cycle dependent manner (12). BAF localizes predominantly in the nucleus during interphase and on chromosomes during mitosis (17, 18). RNA interference (RNAi) experiments reveal that depletion of BAF causes defects of chromatin segregation in \textit{Caenorhabditis elegans} (\textit{C. elegans}) and nuclear envelope assembly in mammalian cells (13, 17, 19). In multicellular eukaryotes, BAF interacts with the LEM-domain family of nuclear proteins, including lamina-associated polypeptide 2 (LAP2), emerin, and MAN1 (12). Most LEM-domain proteins are integral components of the lamina structure at the nuclear periphery and bind to BAF through a highly conserved ~ 40 amino acid residue domain near the N-terminus (20). Since BAF interacts with both DNA and LEM-domain proteins, it has been proposed that the BAF-LEM complexes play a role in chromatin structure and cell cycle progression by molecularly linking chromatin and the nuclear envelope (21). Moreover, BAF has been suggested to regulate gene expression via interaction with LEM-domain proteins and transcriptional repressors (22-24).

Similar to other nuclear lamina-associated proteins, several lines of evidence have revealed that the function of BAF is also regulated by cell-cycle specific phosphorylation (25-27). Phosphoamino acid analyses of BAF have determined N-terminal Ser-4 as a major site and Thr-2 and/or Thr-3 as additional sites of phosphorylation (25, 26). In \textit{Xenopus} egg extracts, Ser-4-phosphorylated BAF is observed throughout cell cycle progression, while BAF is hyperphosphorylated during mitosis (25). In addition, in subcellular fractionation studies of human cells, the unphosphorylated form of BAF was highly enriched in an insoluble pool that contained nuclear matrix and chromatin, whereas equal amounts of phosphorylated BAF were found in the soluble cytoplasmic and nucleoplasmic pool (26). These data suggest the phosphorylation-dependent release of BAF from the chromatin and nuclear envelope during mitosis.

Vaccinia-related kinases (VRKs) are serine/threonine kinases that participate in BAF phosphorylation (26, 27). VRKs were originally discovered by homology to a vaccinia viral kinase called B1 kinase (28-30). In human and murine cells, VRK1, VRK2, and VRK3 compose a family (31). Amongst the VRK family members, VRK1 and VRK2 as well as B1 kinase can catalyze the N-terminal phosphorylation of BAF (26). Most importantly, VRK-mediated phosphorylation causes the loss of DNA binding activity of BAF \textit{in vitro} and the redistribution of BAF in cytoplasmic pools \textit{in vivo} (26). Indeed, RNAi experiments have revealed that elimination of VRK causes defects in association of BAF with chromatin and formation of the nuclear envelope, resulting in arrest of cell cycle progression (27, 32).

Recently, Wiebe and Traktman reported a new function of BAF in poxvirus infection (33). During vaccinia virus replication, BAF directly binds to viral DNA in the cytosol and thereby stalls viral DNA synthesis; vaccinia virus counteracts this impediment to replication by expression of B1 kinase that phosphorylates BAF and disables its DNA-binding activity (33). Given the fact that BAF is also recruited into retroviral PICs at an early phase of retrovirus infection, the report by Wiebe and Traktman suggests a role of cytoplasmic BAF in responding to virus-derived dsDNA in general. However, in contrast to the inhibitory action of BAF during vaccinia virus replication, BAF and its associated DNA-binding activity are beneficial to the integration activity of the retroviral PIC (8, 10, 11, 34). Therefore, these findings raise a possibility that a cellular homologue of B1 kinase, VRK would adversely affect the function of retroviral PICs by...
phosphorylation and subsequent dissociation of BAF. Here, we report that murine VRK1 and VRK2 do indeed induce the dissociation of BAF from MoMLV PICs, resulting in abrogation of intermolecular integration activity in vitro.

**EXPERIMENTAL PROCEDURES**

Cloning of Murine VRK cDNAs - mRNA was purified from either NIH3T3 or L929 cells using an RNeasy Mini kit (Qiagen) and a Poly(A)Purist MAG Kit (Ambion). cDNA synthesis was performed using Accuscript High Fidelity RT-PCR System (Stratagene) according to the manufacturer’s protocol. Open reading frames (ORFs) of murine VRK1, VRK2, and VRK3 (31) were amplified from the cDNA by polymerase chain reaction (PCR) using PfuUltra II Fusion HS DNA Polymerase (Stratagene) and cloned into the Smal site of pGEX-2T bacterial expression vector (GE Healthcare). A catalytic domain mutant, VRK1D177A in which Asp-177 of VRK1 was replaced with alanine (26) was generated from the pGEX-2T encoding wild type (WT) VRK1 using QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Sequences of these plasmid constructs were confirmed with an ABI Prism 3100 genetic analyzer (Applied Biosystems).

Preparation of Recombinant Proteins - To purify glutathione S transferase (GST) tag-fused VRK proteins (GST-VRKs), pGEX-2T constructs were transformed into Escherichia coli BL21(DE3) strain (Stratagene). An overnight culture from a single colony was diluted 1:50 in fresh LB medium containing 100 µg/ml ampicillin and incubated at 37°C. Upon reaching an A_{600} of ~ 0.5, protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM. Bacteria were harvested 5 h after induction and frozen in suspension buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 5 mM DTT), and eluted with protein buffer A containing 15 mM reduced glutathione and 10% (w/v) glycerol. Fractions containing the GST fusion protein were further subjected to gel filtration using a Superdex 75 column (GE Healthcare) with protein buffer B (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol) and stored at -80°C until use. Control GST protein was expressed from pGEX-2T and purified by the same procedure. To prepare hexahistidine-tagged VRK1 (His-VRK1), ORF of murine VRK1 was cloned into the Ndel site of pET15b vector (Novagen). Expression and purification of His-VRK1 were carried out by the same procedure as for GST-VRK proteins. Expression and purification of BAF protein were performed as described previously (8). Concentration of recombinant proteins was quantified using a Coomassie Protein Assay Kit (Pierce).

In Vitro Kinase Assay - Standard assay for BAF phosphorylation was carried out in 15 µl of a reaction containing 500 nM BAF and 500 nM GST-VRKs in kinase buffer A (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 10 mM MgCl$_2$) containing 1 mM ATP and 10 µCi [γ-32P]ATP (3,000 Ci/mmol, PerkinElmer) at 37°C. The reaction was terminated at 5 to 120 min by the addition of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 0.003% bromophenol blue, 0.9% β-mercaptoethanol) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using 15% polyacrylamide gel. Gels were dried and analyzed by a bio-imaging analyzer, BAS 2500 (Fuji Life Science). In particular experiments, BAF phosphorylation was performed with 20 µl of kinase buffer A containing 500 nM BAF, 500 nM GST-VRK1, and 1 mM ATP and analyzed by immunoblotting analysis. Proteins were then separated by SDS-PAGE and transferred to Immobilon P membrane (Millipore). Unphosphorylated and phosphorylated forms of BAF were detected with anti-BAF mouse monoclonal antibody (clone M2, Abnova) and subsequent alkaline phosphatase (AP)-conjugated anti-mouse IgG (Cell Signaling) by using CDP-Star Chemiluminescent Phosphatase Substrate (Kirkegaard & Perry Laboratories).
Signals were analyzed by a luminescence image analyzer, LAS-4000 mini (Fuji Life Science).

**Purification of Hyperphosphorylated BAF** - An *in vitro* kinase assay using 2.5 µM BAF and 2.5 µM GST-VRK1 was performed in 1 ml of kinase buffer A containing 1 mM ATP at 37°C for 2 h. Hyperphosphorylated form of BAF (P-BAF) was then purified by gel filtration through a Superdex 75 column in protein buffer C (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 10% glycerol).

**Gel Mobility Shift Assay** - A ScaI/NdeI fragment of pUC19 (691-bp) was used as a short DNA substrate for the gel mobility shift assay. BAF/DNA complex was formed with 250 nM BAF and 250 pM substrate DNA in 50 µl of BAF binding buffer (20 mM Hepes-NaOH, pH 7.5, 1 mM DTT, 100 ng/ml BSA) at 30°C for 1 h. To evaluate the effect of VRK1 on the BAF/DNA complex, 5 µl of the reaction was mixed with 0.2 to 5 nM GST-VRK1 in 25 µl kinase buffer containing 20 mM Hepes-NaOH, pH 7.5 and 1 mM ATP and incubated at 37°C for 1 h. The mixture was separated by agarose gel electrophoresis with a 0.4% agarose gel in Tris-acetate-EDTA (TAE) buffer, and BAF/DNA complex was blotted to a GeneScreen Plus membrane (PerkinElmer). Southern blotting analysis to detect the substrate DNA was performed by AP-labeled substrate DNA probe and Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare) followed by exposure to BioMax Light Film (Kodak).

**BAF Release Assay** - To prepare DNA substrate, 5'–biotinylated 500-bp fragment was amplified from ΦX174 DNA (New England Biolabs) by PCR and purified with a QIAquick PCR purification kit (Qiagen). One microliter of the biotinylated DNA was immobilized with 20 µl of Dynabeads M-280 Streptavidin (Dynabeads kilobaseBINDER Kit, Invitrogen) according to manufacturer's protocol and resuspended with 20 mM Hapes-NaOH, pH 7.5. Dynabeads-conjugated DNA (30 µM) was then incubated with 500 nM BAF in 50 µl of BAF binding buffer at 30°C for 1 h. The BAF/DNA complex was washed with 400 µl of BAF binding buffer containing 30 mM NaCl three times using magnetic stand and treated with 500 nM GST-VRK1 in 20 µl of kinase buffer A containing 1 mM ATP at 37°C for 60 min. Beads complex was washed with 200 µl of washing buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM EDTA, 0.01% NP-40), and BAF in fractions bound to DNA and released from DNA were detected by immunoblotting analysis using anti-BAF monoclonal antibody. In certain experiments, BAF/DNA complex was treated with 100 nM GST-VRK1, -VRK2, and -VRK3 in the presence of 1 mM ATP at 37°C for 1 and 60 min.

**Velocity Sedimentation Assay of BAF/DNA Complex** - Long DNA substrate used for the velocity sedimentation assay was ΦX174 DNA linearized with XhoI (5,386-bp). To form nucleoprotein complexes, 10 nM BAF and 0.1 pM substrate DNA were incubated in 100 µl of the reaction at 30°C for 1 h. Then, 20 µl of a mixture (20 mM Hapes-NaOH, pH7.5, 10 mM MgCl₂, 150 mM NaCl, 1 mM ATP) containing 100 nM GST-VRK1 or GST (or no GST fusion protein) was added, and the reaction was further incubated at 37°C for 1 h. Two milliliter of a continuous sucrose gradient was made by layering 15, 20, 25, and 30% sucrose solutions in gradient buffer (20 mM Hepes-NaOH, pH 7.5, 5 mM MgCl₂, 150 mM KCl, 6 mM EDTA) at 4°C overnight. The gradient was overlayed with the BAF/DNA complex mixture and centrifuged at 30,000 rpm at 4°C for 1 h with a Beckman TLS55 rotor. The gradient was fractionated from the top into 10 fractions, and each fraction was deproteinized by incubation with 5 mg/ml proteinase K and 1% SDS at 37°C for 1 h. Substrate DNA in each fraction was isolated by phenol/chloroform extraction and ethanol precipitation, and detected by Southern blotting using AP-labeled DNA probe for ΦX174 DNA sequence and Gene Images AlkPhos Direct Labeling and Detection System.

**Preparation of MoMLV PICs** - NIH3T3 cells and MoMLV-producing cell line, clone 4 (2) were grown in Dulbecco’s modified Eagle medium containing high glucose and sodium pyruvate (Sigma) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 10% fetal calf serum, and GlutaMAX Supplement I (Invitrogen). MoMLV PICs were prepared by coculture of NIH3T3 cells and clone 4 cells as described previously (2). Briefly, cytoplasmic extract from cocultured cells containing PICs was isolated with buffer A (20 mM Hapes-NaOH, pH 7.5, 5 mM MgCl₂, 150 mM NaCl, 1 mM ATP) and centrifuged at 37°C for 60 min. Beads complex was washed with 200 µl of washing buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM EDTA, 0.01% NP-40), and BAF in fractions bound to DNA and released from DNA were detected by immunoblotting analysis using anti-BAF monoclonal antibody. In certain experiments, BAF/DNA complex was treated with 100 nM GST-VRK1, -VRK2, and -VRK3 in the presence of 1 mM ATP at 37°C for 1 and 60 min.
KCl, 10 mM DTT) containing 20 µg/ml aprotinin (Sigma) and 0.025% digitonin and stored at -80°C in buffer B (buffer A containing 6 mM EDTA and 6% sucrose). The PIC sample was gel filtrated through a spin column of Sephacryl S-1000 Superfine (GE Healthcare) equilibrated with buffer B containing 0.1% BSA before use (initial PIC) (10). To prepare high-salt-treated MoMLV PICs, the initial PIC fraction was incubated on ice in the presence of 750 mM KCl for 1 h and then gel filtrated on a Sephacryl S-1000 Superfine spin column equilibrated with buffer B containing 750 mM KCl and 0.1% BSA, followed by a second gel filtration step with a Sephacryl S-1000 Superfine spin column equilibrated with buffer B containing 150 mM KCl and 0.1% BSA (salt-stripped PIC) (10).

Immunoprecipitation of PICs - A volume of 50 µl of initial PIC was incubated with 500 nM GST-VRK1 or GST in 100 µl of kinase buffer B (20 mM Heps-NaOH, pH 7.5, 10 mM MgCl₂, 150 mM KCl, 1 µM ATP, 1 mM DTT, 0.04% BSA) at 37°C for 1 h. For the immunoprecipitation assay of salt-stripped PIC, 50 µl of high-salt-treated PIC was diluted in 100 µl of kinase buffer B without GST fusion protein prior to the assay. Reactions were mixed with 400 µl of buffer C (20 mM Heps-NaOH, pH7.5, 5 mM MgCl₂, 150 mM KCl, 6 mM EDTA, 0.04% BSA, 0.1% NP-40, 20 µg/ml aprotinin) and 5 µg of anti-BAF rabbit IgG (10) or control rabbit IgG and incubated at 4°C for 1 h. After adding 40 µl of protein A/G agarose beads (Santa Cruz Biotechnology), incubation was continued at 4°C for 2h. The immune complex was washed three times with buffer C and then deproteinized by proteinase K and SDS. Viral DNA was recovered from the complex by phenol/chloroform extraction and ethanol precipitation, suspended in TE buffer containing 20 µg/ml RNase A (Qiagen), and detected by Southern blotting analysis using AP-labeled DNA probe for MoMLV LTR sequence (7). In a particular experiment, initial PIC was treated with kinase buffer B containing 1 mM ATP, GTP, dATP, or CTP in the absence of GST fusion protein, and subjected to immunoprecipitation analysis using anti-BAF antibody.

Velocity Sedimentation Assay of PICs - Pretreatment of PICs with VRK1 was performed in 200 µl of kinase buffer B containing 100 µl of initial PIC, 500 nM GST-VRK1 or GST in the presence of 1 mM ATP for 1 h on ice. The reactions and an equal volume of a reaction containing 100 µl of salt-stripped PICs but not containing GST protein and ATP, were loaded on 15 to 30% sucrose gradients in gradient buffer and subjected to velocity sedimentation assay as described previously (10). Viral DNA was detected by Gene Images AlkPhos Direct Labeling and Detection System.

Integration Activity Assay - Activity of the MoMLV PICs was evaluated by the previously described in vitro integration activity assay using ΦX174 DNA as a target DNA (2, 7, 8). Prior to the assay, 20 µl of initial PIC was incubated with 20 to 500 nM GST-VRK1, GST-VRK1D177A, or GST in 100 µl of kinase buffer B containing 1 µM ATP at 37°C for 1 h. Integration reactions were carried out in 200 µl of reaction mixture containing 20 mM Heps-NaOH, pH7.5, 10 mM MgCl₂, 150 mM KCl, 10 mM DTT, 0.02% BSA, 15% (w/v) glycerol, and 1.4 nM ΦX174 replicative form 1 DNA (New England Biolabs) at 37°C for 30 min. After the incubation, PICs were deproteinized with 1 mg/ml proteinase K and 1% SDS at 37°C for 1 h, and then DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The reaction products were digested with BamHI and then separated by agarose gel electrophoresis with a 0.8% agarose gel. After Southern blot transfer, integration products were detected by an AP-labeled DNA probe for MoMLV LTR sequence and Gene Images AlkPhos Direct Labeling and Detection System. Integration activities were quantified with MultiGauge software (Fuji Life Science) by measuring the density of bands of intermolecular integration products, autointegration products, and the unreacted viral DNA. Intermolecular integration and autointegration activities were calculated as a percentage of total viral DNA (intermolecular, autointegration, and unreacted DNA) converted into intermolecular integration and autointegration products, respectively. In particular experiments, pretreatment of the PICs in kinase buffer B was performed i) with 500 nM GST-VRK1 in the presence of 100 µM staurosporine (Sigma) and absence of added ATP, ii) with 500 nM GST-VRK1 in the presence of 100 µM staurosporine (Sigma) and absence of added ATP.
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GST-VRK1, -VRK2, and -VRK3, iii) with or without GST-VRK1 in the presence of 1 to 1000 µM ATP, or iv) without GST fusion protein in the presence of 1 mM nucleoside triphosphate (NTP: ATP, GTP, CTP, or UTP) or deoxynucleoside triphosphate (dNTP: dATP, dGTP, dCTP, dTTP, or dUTP).

Reconstitution of initial and salt-stripped PICs with 10 and 100 nM unphosphorylated or hyperphosphorylated form of BAF were carried out as previously described for the autointegration barrier reconstitution assay (8).

Isolation of PICs from VRK1-knockdown cells - The siRNA (stealth RNAi, invitrogen)-targeting sequences against murine VRK1 was 5'-CGGUACUGCCCAGAUGGAGUUCAUA-3' and a nonspecific control stealth RNAi (medium GC duplex) was purchased from invitrogen. A total of 200 pmol of stealth RNAi was used to transfect 1 x 10^5 of NIH3T3 cells by using 2 µl of Lipofectamine RNAiMAX (Invitrogen). The siRNA transfections were performed twice in two consecutive days. On third day, transfected cells were cultured with 5 ml of supernatant from clone 4 cells in the presence of 8 µg/ml polybrene for 7 h. Cytoplasmic extract was prepared from the virus-infected cells using 100 µl of buffer A containing digitonin and stored in buffer B.

For immunoblotting of endogenous VRK1, a rabbit serum was generated against His-VRK1. Following IgG purification from the antisera using a HiTrap Protein G HP column (GE Healthcare), specific antibody was isolated by negative affinity purification with GST-VRK2 and -VRK3, and subsequent positive affinity purification with GST-VRK1. To detect endogenous VRK1, cytoplasmic extract of MoMLV-infected NIH3T3 cells that had been transfected with siRNA, was lysed in SDS sample buffer and resolved by SDS-PAGE using 12% polyacrylamide gel. Proteins were detected by immunoblotting analysis using the anti-VRK1 rabbit polyclonal IgG and subsequent AP-conjugated anti-rabbit IgG (Cell Signaling).

RESULTS

Murine VRK1 Disrupts BAF/DNA Complex - Nichols et al. have reported that human VRK1 phosphorylates the N’-terminus of BAF, and this phosphorylation results in a loss of DNA-binding activity in vitro (26). However, it remains unclear whether this VRK-mediated phosphorylation also suffices for dissociation of BAF from a nucleoprotein complex. In an attempt to test this in vitro, murine VRK1 was generated as a recombinant protein. In cultured murine cells, three differentially spliced isoforms for VRK1s, which was named as VRK1ΔΔ, is most similar to human VRK1 (31), we chose this splicing variant for subsequent analysis. GST-tag fused VRK1 (GST-VRK1) was purified from bacterial cells and checked for its activity by an in vitro kinase assay (Fig. 1A). In the assay using [γ-32P]ATP, the phosphorylated form of BAF was visualized by autoradiography (Fig. 1A, top panel) and in parallel, an assay using cold ATP, phosphorylated BAF as well as substrate (unphosphorylated) BAF (10 kDa) were detected by immunoblotting analysis with an anti-BAF antibody (Fig. 1A, bottom panel). As shown in Fig. 1A, the recombinant murine VRK1 displayed rapid phosphorylation activity of BAF in the presence of 1 mM ATP and 10 mM MgCl2. A previous study has demonstrated that VRK1-mediated phosphorylation of BAF shows a biphasic profile; an initial phase represents phosphorylation of the N-terminal Ser-4 and a second slow phase represents additional phosphorylation of Thr-2 and/or Thr-3 (26). Likewise in our assay, a single shifted species of BAF (hypophosphorylated form) was observed after ~ 5 min (Fig. 1A, gray arrowhead) and a double shifted species (hyperphosphorylated form) subsequently accumulated upon further incubation with GST-VRK1 (Fig. 1A, white arrowhead).

By using a gel mobility shift assay, we next evaluated whether VRK1 could induce removal of BAF from DNA complexes. Simple nucleoprotein complex was formed with BAF and short DNA (691-bp) and then the complex was incubated with GST-VRK1 in the presence of 1 mM ATP and 10 mM MgCl2. The electrophoretic mobility of the substrate DNA was monitored by Southern blotting analysis. As shown in Fig. 1B, the BAF/DNA complex could be observed as a slowly migrating complex in agarose gel electrophoresis (Fig. 1B, lanes 1 and 2). However, the complex disappeared following GST-VRK1 treatment (Fig. 1B, lanes
ATP (Fig. 1D) in control reactions with GST-VRK1 in absence of change in the sedimentation rate was not observed free DNA (Fig. 1D) indistinguishable sedimentation rate from that of GST-VRK1, but not with GST, resulted in an treatment of this BAF/DNA complex with gradient (Fig. 1D) than substrate DNA alone in a 15-30% sucrose 5,386-bp) formed a complex with faster mobility BAF and long DNA (linearized BAF complex with longer DNA. In this assay, assay to confirm the ability of VRK1 to disrupt the DNA is mediated by BAF phosphorylation. that VRK1-induced dissociation of BAF from MoMLV PIC, resulting in the disruption of BAF/DNA complex by GST-VRK1 was dependent on its kinase activity.

To ascertain whether the VRK1-mediated dissociation of BAF involves phosphorylation, BAF/DNA complex was formed with short DNA (500-bp) that had been conjugated with magnetic beads, and after treatment with GST-VRK1, BAF in DNA-bound (B) and released (R) fractions were detected by immunoblotting analysis with an anti-BAF antibody (Fig. 1C). The results showed that initial BAF bound to the DNA was in an unphosphorylated form (Fig. 1C, lanes 1 and 2), whereas the phosphorylated form of BAF was observed in the released fraction from a reaction in which BAF was treated with GST-VRK1 in the presence of ATP (Fig. 1C, lanes 3 and 4). Even with GST-VRK1, this phosphorylation-mediated release of BAF was not observed in the absence of ATP (Fig. 1C, lanes 5 and 6). In addition, neither incubation of BAF/DNA complex with GST alone or with GST-VRK1D177A, a catalytic domain mutant of VRK1 involved in coordinating ATP (26) released BAF (Fig. 1C, lanes 7-10), indicating that VRK1-induced dissociation of BAF from DNA is mediated by BAF phosphorylation.

We also performed a velocity sedimentation assay to confirm the ability of VRK1 to disrupt the BAF complex with longer DNA. In this assay, BAF and long DNA (linearized ΦX174 DNA, 5,386-bp) formed a complex with faster mobility than substrate DNA alone in a 15-30% sucrose gradient (Fig. 1D, panels 1 and 2) (35). In contrast, treatment of this BAF/DNA complex with GST-VRK1, but not with GST, resulted in an indistinguishable sedimentation rate from that of free DNA (Fig. 1D, panels 3 and 4), indicating that BAF was dissociated from the DNA complex. This change in the sedimentation rate was not observed in control reactions with GST-VRK1 in absence of ATP (Fig. 1D, panel 5). Taken together, these data demonstrated that VRK1 has the ability to dissociate BAF from DNA via phosphorylation of BAF, resulting in the disruption of BAF/DNA complex in vitro.

**VRK1 Induces Dissociation of BAF from the MoMLV PIC** - We next examined if VRK1 could dissociate BAF from MoMLV PICs as in the case of simple BAF/DNA complex. In order to test this, a cytoplasmic fraction containing PICs was isolated from MoMLV-infected cells and subjected to immunoprecipitation using an anti-BAF antibody. MoMLV DNA was then extracted from PICs captured by protein A/G agarose and detected by Southern blotting analysis (10). Our previous study has shown that recovery of PICs by this immunoprecipitation with anti-BAF antibody was significantly impaired by treatment with high-salt concentration (salt-stripping), indicating removal of BAF from the PIC (10). When PICs were incubated with GST-VRK1 in the presence of 1 µM ATP prior to immunoprecipitation, anti-BAF antibody did not precipitate the PICs, similar to the result with high-salt treatment (Fig. 2A). In contrast, pretreatment with control GST protein had no effect on the efficiency of immunoprecipitation of PICs (Fig. 2A).

To gain further insight into the VRK1-mediated dissociation of BAF, we compared the sedimentation properties of initial (untreated) and VRK1-treated PICs in sucrose gradients (Fig. 2B). It has been proposed that BAF compacts the viral DNA within PIC by its DNA-bridging property, thereby making the viral DNA inaccessible as a target for autointegration (8, 10). As shown in Fig. 2B, PICs treated with GST-VRK1 (panel 2) sedimented more slowly than PICs treated with control GST (panel 1) in the sucrose gradient, and this was similar to the effect of salt-stripping (panel 3), indicating that the viral DNA adopts a more open structure upon BAF dissociation (10). These data demonstrate that VRK1 can dissociate BAF from the MoMLV PIC in vitro.

**Integration activity of MoMLV PIC is inhibited by VRK1** - Removal of BAF from PIC by high-salt treatment causes self-destruction of viral DNA by autointegration, thereby diminishing intermolecular integration activity of the PIC in vitro (7, 10). To test whether VRK1-mediated dissociation of BAF also induces dysfunction of the MoMLV, in vitro PIC integration assays were performed in combination with VRK1 treatment (Fig. 3A). PICs were incubated with GST-VRK1 in the presence of 1 µM ATP and then subjected to the in vitro integration activity assay. As we expected, quantification of integrated products revealed that intermolecular integration activity of
the PIC was significantly decreased by treatment with GST-VRK1 and autointegration increased (Fig. 3A, lanes 2-4), similar to the effect of high-salt treatment (Fig. 3A, lane 9). On the other hand, control GST and GST-VRK1D177A proteins had no effect on the activities of PICs (Fig. 3A, lanes 5-8). In order to determine whether the VRK1-mediated inhibition of integration activity was dependent on its kinase activity, we used staurosporine, a kinase inhibitor that was reported to decrease autophosphorylation and casein phosphorylation activities of VRK1 in vitro (29). When pretreatment of PICs with GST-VRK1 was carried out in the presence of staurosporine, there was little inhibition of intermolecular integration activity and no increase of autointegration activity (Fig. 3B, lanes 2 and 3). The staurosporine treatment did not show nonspecific stimulation of integration activity of the PICs (Fig. 3B, lanes 4 and 5). These results suggest that VRK1 abrogates the function of the PICs by its kinase activity.

**VRK1-mediated phosphorylation disables function of BAF in the PIC** - As described above, treatment with VRK1 elicited dissociation of BAF from PICs (Fig. 2) and inhibition of intermolecular integration activity of the PICs (Fig. 3). Given the evidence that disruption of simple BAF/DNA complexes by VRK1 involves BAF phosphorylation (Fig. 1C), we speculated that phosphorylation of BAF within PICs is a prerequisite for the BAF dissociation and subsequent inhibition of PIC activity. However, direct analysis of the phosphorylation status of BAF within the PICs is not possible because the abundance of PICs in cytoplasmic extract is too low to directly detect BAF by immunoblotting analysis. In order to demonstrate that phosphorylation inactivates the function of BAF in the PIC, an in vitro kinase assay using BAF and GST-VRK1 was carried out, followed by gel filtration purification of the resultant hyperphosphorylated form of BAF (P-BAF). Immunoblotting analysis using an anti-BAF antibody confirmed that all of the substrate BAF (Fig. 4A, left lane) was converted to P-BAF (Fig. 4A, right lane). Then, the P-BAF was subjected to in vitro integration activity assays. As observed in previous studies (8, 10, 35), unphosphorylated BAF efficiently stimulates intermolecular integration activity of initial PICs (Fig. 4B, lanes 1-3) and restored the activity to salt-stripped PICs (Fig. 4B, lanes 6-8), accompanied by a reduction of autointegration activity. In contrast, incubations of initial and salt-stripped PICs with P-BAF resulted in neither stimulation nor restoration of the intermolecular integration activities (Fig. 4B, lanes 4, 5, 9, and 10). This data indicates that BAF loses its functional activity in in vitro integration assays upon phosphorylation by VRK1.

**Activities of VRK Family Proteins in Dysfunction of MoMLV PICs** - The mammalian VRK family is comprised of three members, VRK1, VRK2, and VRK3 (31). Although all the VRK family proteins share a high degree of identity in their catalytic domains, each VRK has distinctive character in enzymatic activity and intracellular localization (31). We therefore compared their ability to disrupt PIC function. To assess in vitro activities, murine VRK2 and VRK3 were also prepared as GST-fusion proteins (Fig. 5A) and used in in vitro kinase assays for phosphorylation of BAF using [γ-32P]ATP. A previous study has described that, unlike enzymatic activities of VRK1 and VRK2, human and mouse VRK3 were catalytically inert as measured by autophosphorylation and casein phosphorylation in vitro (31). We find that GST-VRK2 as well as GST-VRK1 had activity to phosphorylate BAF in vitro, and the major phosphorylation state was hyperphosphorylated (Fig. 5B, white arrowheads). However, contrary to a previous report (31), we observed incorporation of 32P into BAF by the incubation with GST-VRK3, indicating that VRK3 is an active kinase (Fig. 5B), albeit a less efficient one. In addition, the reaction only produced the hypophosphorylated form of BAF (Fig. 5B, gray arrowheads).

In order to test whether the kinase activities of the VRK family proteins are capable of disrupting simple BAF/DNA complex, we performed the BAF release assay with GST-VRK1, -VRK2, and -VRK3. At an early time point (1 min), dissociation of BAF was observed in reactions treated with GST-VRK1 and -VRK2 (Fig. 5C, top panel, lanes 2 and 4). Then, most of BAF was released as the hyperphosphorylated form at a later time point (60 min) of the GST-VRK1 and -VRK2 treatments (Fig. 5C, bottom panel, lanes 2 and 4). In contrast to the activity of VRK1 and VRK2,
some BAF was observed in the released fraction following 60 min treatment with GST-VRK3 (Fig. 5C, bottom panel, lane 6). Interestingly, this fraction contained two electrophoretically distinct forms of BAF, which was similar to the BAF present in released fractions at early time point of GST-VRK1 and -VRK2 treatments (Fig. 5C, top panel, lanes 2 and 4). An additional BAF release assay using \([\gamma-^{32}\text{P}]\text{ATP}\) revealed that incorporation of \(^{32}\text{P}\) was detected in the two forms of BAF in the released fraction after treatment with all GST-VRKs (data not shown). This result indicates that although phosphorylation is a prerequisite for dissociation of BAF from DNA by all VRK family proteins, VRK1 and VRK2 are able to disrupt the simple BAF/DNA complexes more efficiently than VRK3.

We then assessed abilities of VRK family proteins to inhibit intermolecular integration activity of the PICs in vitro. In parallel to the activity to disrupt BAF/DNA complexes (Fig. 5C), pretreatment of PICs with GST-VRK1 and -VRK2 reduced intermolecular integration activity with a concomitant increase of autointegration activity (Fig. 5D, lanes 2 and 3), whereas treatment with GST-VRK3 did not affect integration activity (Fig. 5D, lane 4). Note that level of inhibition of intermolecular integration activity by GST-VRK1 was reproducibly stronger than that by GST-VRK2 with several batches of recombinant proteins (data not shown). Taken together, these results demonstrate that amongst VRK family proteins, VRK1 and VRK2 abolish the function of MoMLV PICs in vitro.

Implications of Endogenous VRK1 in Disruption of the PIC Activity - The above experiments showing VRK1- and VRK2-mediated inhibition of PIC integration activity were carried out with reactions containing 1 µM of exogenous ATP (Fig. 2, 3, and 5). In the course of performing the experiments, we noted that, even without added VRK proteins, intermolecular integration activity of MoMLV PICs was also inhibited by addition of higher concentrations (100 and 1,000 µM) of ATP (Fig. 6A). Similar to the effects of VRK1- and high-salt-treatment, autointegration activity of PICs was increased by the ATP treatment (Fig. 6A) (36). To ascertain whether all nucleoside triphosphates could induce the disruption of the PIC function, we carried out in vitro integration activity assays using PICs that were pretreated with 1 mM NTP (ATP, GTP, CTP, or UTP) and dNTP (dATP, dGTP, dCTP, dUTP, or dTTP). The results showed that robust inhibition of intermolecular inhibition was observed by the addition of dATP as well as ATP (Fig. 6B, lanes 2 and 6). Likewise, treatment with GTP caused a slight but reproducible inhibition of PIC activity (Fig. 6B, lane 3). However, other nucleotides did not affect integration activity of the PIC (Fig. 6B).

In order to confirm that the nucleotide-induced inhibition of PIC integration activity involved BAF dissociation, immunoprecipitation analysis using anti-BAF was conducted. As shown in Fig 6C, recovery of PICs in immunoprecipitation assays was significantly impaired following ATP and dATP treatments. Whereas the efficiency of immunoprecipitation was also modestly decreased with GTP treatment, this impairment was not observed with control treatment with CTP (Fig. 6B and C).

It should be noted that ATP and GTP are the two main donors of phosphate group in most of kinase reactions including VRK (37). In addition, when we carried out in vitro kinase assay, GST-VRK1 catalyzed BAF phosphorylation with GTP and dATP as well as ATP but not with other nucleoside triphosphates (data not shown). Our data therefore suggest the presence of endogenous kinase(s) in cytoplasmic extract, which induce disruption of the PIC integration activity through dissociation of BAF. To determine if VRK1 is involved in the ATP-induced abolishment of the PIC activity, endogenous VRK1 was depleted from the cytoplasm of MoMLV-infected NIH3T3 cells by siRNA-based knockdown technique (Fig. 6D). We next isolated PICs from the cells and assessed whether the ATP treatment still inhibited intermolecular integration activity of the PIC. As shown in Fig. 6E, PICs derived from VRK1-knockdown cells remained capable of intermolecular integration even in the presence of added ATP (lanes 3 and 4), whereas the PICs from mock- and control siRNA-transfected cells lost their integration activity by the ATP treatment (lanes 1, 2, 5, and 6). This result indicates that endogenous VRK1 is a cytosolic factor responsible for the disruption of PIC activity, which is activated by addition of ATP.
DISCUSSION

Posttranslational modifications including phosphorylation play an important role in regulating many cellular processes. Protein phosphorylation is often carried out by cellular and viral kinases during viral infection to modulate productive infection (38). In the case of vaccinia virus infection, a viral serine/threonine kinase called B1 kinase counteracts an inhibition caused by BAF during viral DNA replication via phosphorylation of the cellular protein, thereby enabling productive infection (33). In this study, we have obtained evidence that BAF phosphorylation adversely affects the function of another type of virus; VRK1 and VRK2, cellular homologues of B1, were capable of disrupting the MoMLV PIC activity by phosphorylation and dissociation of BAF in vitro.

Phosphorylation of BAF is cell cycle specific (25, 26). BAF phosphorylation has also been reported to regulate the interaction of BAF with its binding partners including dSDNA and LEM proteins (25, 26). A previous study using a DNA cellulose affinity resin revealed that the VRK1-mediated phosphorylation reduces the affinity of BAF for DNA, resulting in inhibition of subsequent nucleoprotein complex formation with substrate DNA (26). In this study, we have addressed the question whether, once BAF forms a complex with DNA, VRK1 treatment could release BAF from the DNA complex. Our results showed that BAF phosphorylation suffices to disrupt the simple BAF/DNA complex in vitro (Fig. 1). Since it has been proposed that the DNA-binding activity of BAF accounts for its incorporation into the retroviral PIC through direct association with viral DNA (10), we next hypothesized that treatment of PICs with VRK1 could induce removal of BAF. Immunoprecipitation analysis using anti-BAF IgG revealed that BAF was indeed dissociated from the PICs following VRK1 treatment (Fig. 1). Since it has been proposed that viral DNA is protected against autointegration by BAF-mediated compaction (8, 10). In the present study, VRK1 treatment also induced the opening of viral DNA in the PICs, which was indistinguishable from the salt-stripped PICs (Fig. 2B). In the case of high salt-treatment, high ionic strength likely strips a number of protein components including BAF from the PIC, whereas VRK1 specifically induces removal of BAF from the PIC. Based on these results, we conclude that BAF is a factor solely responsible for the compaction of viral DNA within the PIC. However, note that BAF is not likely phosphorylated only while it’s bound to DNA; reasonable interpretation of VRK1-mediated BAF/DNA disruption would be that the kinase also phosphorylates BAF in the free state preventing rebinding and thereby leading to depletion of the bound state.

In addition to the VRK1-induced dissociation of BAF, we found that treatment of MoMLV PICs with VRK1 resulted in significant inhibition of intermolecular integration activity in vitro (Fig. 3). When the VRK1 treatment was performed in the presence of staurosporine, a well-characterized kinase inhibitor, the inhibitory effect of VRK1 in PIC activity was not observed (Fig. 3B). Although we could not directly determine the phosphorylation state of BAF within the PIC before and after VRK1 treatment due to the low abundance of PICs in cytoplasmic fractions, this data indicates that the kinase activity of VRK1 is integral to the dissociation of BAF and subsequent disruption of the PIC integration activity. Supporting this, VRK1-mediated phosphorylation inactivated the function of BAF in integration activity of PICs (Fig. 4).

In our study, treatments of PICs with GST-VRKs were mostly performed in the presence of 1 μM ATP (Fig. 2, 3, and 4). In the case of VRK1, abolishment of intermolecular integration activity was observed even when the PIC was incubated with high concentration (500 nM) of GST-VRK1 in the absence of exogenous ATP, whereas the addition of 1 μM (or 10 μM) ATP was required for treatment with lower concentration (20 and 100 nM) of GST-VRK1 (Fig. 3). Considering the previous data that the $K_m$ of VRK1 for ATP is 50 μM (37), the concentration of exogenous ATP used in the PIC experiments seems to be low to activate VRK1, but the level of ATP in the PIC samples as measured by a luciferase-based luminescent assay.
was much lower (0.315 ± 0.142 µM). It would be therefore reasonable to use 1 to 10 µM of exogenous ATP for the treatment of PIC with VRKs to obtain reproducible results. However, it should be noted that treatment with higher concentrations of ATP (100 to 1,000 µM) also inhibited intermolecular integration activity of PICs even in the absence of added GST-VRK1 as discussed below (Fig. 6).

The VRKs are a novel kinase family belonging to the casein kinase 1 (CK1) group (39). Amongst the VRK family proteins, VRK1 is the best-characterized protein, and has been shown to phosphorylate not only BAF but also histones and several transcription factors including p53 (26, 39-44). VRK2 also catalyzes the phosphorylation of BAF and p53 (26, 45). In contrast to VRK1 and VRK2, VRK3 is the least-characterized member of VRK family and has been reported to be catalytically inert as measured by its autophosphorylation and casein phosphorylation in vitro (31). Structural comparison of VRK2 and VRK3 demonstrates that the overall folding of VRK3 is very similar to that of VRK2 and the differential activities between the two VRKs cannot be explained by any major structural differences but VRK3 contains several disruptions in key motifs, which would lead to the loss of kinase activity (46). In addition, a recent report reveals that the function of extracellular signal regulated kinase (ERK) is inhibited by VRK3 through direct interaction with a phosphatase of ERK called vaccinia H1-related (VHR) (47). Since the activation of VHR by VRK3 does not likely involve phosphorylation, this result suggests that VRK3 acts as a scaffolding modulator in regulation of the ERK signaling pathway as seen in other enzymatically inactive kinases (46, 47). However, our in vitro kinase assay to assess incorporation of 32P showed that, when BAF was used as a substrate, VRK3 does have an ability to catalyze hypophosphorylation of BAF (Fig. 5B).

In addition, the in vitro kinase assay using BAF mutants, in which N-terminal Thr-2, Thr-3, and/or Ser-4 had been substituted with alanine, revealed that Ser-4 is the sole site for VRK3-mediated phosphorylation (data not shown). In contrast to the kinase activity of VRK3, VRK1 and VRK2 catalyzed hyperphosphorylation of BAF (Fig. 5B) as shown by a previous study (26). Regarding the target residues of VRK1 and VRK2 for BAF hyperphosphorylation, Nichols et al. have described that Ser-4 undergoes initial phosphorylation and subsequent phosphorylation takes place on Thr-2 and/or Thr-3 (26). Unexpectedly, when simple BAF/DNA complex was used as a substrate, hyperphosphorylation as well as hypophosphorylation occurred by VRK3 treatment, which was similar effect by VRK1 and VRK2 treatments at early time point (Fig. 5C). However, the VRK3 treatment did not result in complete dissociation of BAF from DNA (Fig. 5C). This indicates that, although VRK3-mediated phosphorylation may weaken BAF-DNA interaction, the kinase activity is insufficient to ablate BAF association from DNA. Importantly, in parallel to the activity to disrupt simple BAF/DNA complex, inhibition of intermolecular integration activity of the PICs was observed with VRK1 and VRK2 but not with VRK3 (Fig. 5D). Therefore, our results suggest that, although phosphorylation is definitely the first process required for removal of BAF from DNA, VRK1 and VRK2 may possess additional activity such as higher affinity for BAF than VRK3, leading sufficient phosphorylation and complete dissociation of BAF, which ultimately results in abolition of PIC function.

A question that arises from the results of our study is; do VRK1 and VRK2 affect the in vivo integration activity and replication of retroviruses? One approach to address this question would be the use of small interfering RNA (siRNA)-based knockdown for the VRKs. However, VRK1 has been shown to be an early response gene that is implicated in normal cell proliferation and division (32). Consistent with previous reports (32, 48), our knockdown experiments showed that depletion of endogenous VRK1 in NIH3T3 cells caused severe inhibition of cell proliferation. Because MoMLV requires cell division to replicate (49), this fact makes it difficult to evaluate the role of VRK in virus infection. On the other hand, our in vitro data presented here suggest a role of endogenous kinases in disruption of PIC function; even without exogenous VRKs, inhibition of intermolecular integration activity was elicited by addition of nucleotides such as ATP, GTP, and dATP into PIC samples (Fig. 6A and B). This nucleotide-induced inhibition accompanied the enhancement of autointegration activity (Fig. 6A and B). Supporting this data, Farnet and Haseltine...
have shown that treatment of HIV-1 PICs with nucleoside triphosphates including ATP induces the formation of circular viral DNA, which is generated by simple end-to-end joining and autointegration \textit{in vitro} (36). This previous observation is quite consistent with our results, and as Farnet and Haseltine have proposed, the ability of NTPs to inhibit intermolecular integration and enhance autointegration may be a general feature of retroviral PICs (36). However, more important data obtained by our study is that pretreatment of MoMLV PICs with ATP, GTP, and dATP induced dissociation of BAF (Fig. 6C) and these nucleotides can be utilized as phosphate donors for BAF phosphorylation by VRK1 (data not shown). This data suggests that certain kinase(s) in cytoplasmic fractions from MoMLV-infected NIH3T3 cells phosphorylate BAF within the PIC, resulting in BAF dissociation and subsequent inhibition of the PIC integration activity. It has been reported that both VRK1 and VRK2 are ubiquitously expressed in various tissues (28), however they show distinct patterns of subcellular localization. VRK1 has a nuclear localization signal in the C-terminal domain and it is mostly detected in the nucleus, but is also found in the cytoplasm in some cell lines as well (31, 41, 50, 51). On the other hand, VRK2, which lacks a putative nuclear localization signal, is observed in cytosol, particularly associating with the endoplasmic reticulum (ER) and mitochondria (31, 45). Therefore, given the available data, endogenous VRK1 and VRK2 in cytoplasm may be the kinase that causes dysfunction of PIC through dissociation of BAF upon incubation with ATP, GTP, and dATP (Fig. 6). Indeed, when the PIC was isolated from VRK1-knockdown cells, ATP-induced inhibition of intermolecular integration did not occur (Fig. 6E). Thus, we conclude that at least endogenous VRK1 is a cytosolic factor that induces disruption of the PIC activity through dissociation of BAF, which is activated under certain conditions \textit{in vitro}. Interestingly, a recent study revealed that cytosolic VRK1 is likely to be inactivated by direct interaction with the GDP-bound form of Ran (RanGDP), suggesting that the kinase activity of VRK1 is spatially regulated by its subcellular distribution (50). Although further analysis will be required to address whether the PIC activity is modulated by VRK1/2 or other kinases, it can be proposed to be an intrinsic inhibitory mechanism for the retroviral PIC integration.

While \textit{in vitro} functions of BAF in the stimulation of intermolecular integration and suppression of autointegration have been well documented with HIV-1 and MoMLV PICs (8, 9), the \textit{in vivo} role of BAF during retroviral replication is ambiguous (15, 16). A recent study reports the SET complex, an ER-associated DNA repair complex as a barrier to autointegration in HIV-1 infection (52). However, all the data analyzing the \textit{in vivo} role of BAF were obtained by knockdown experiments using siRNA technology (15, 16, 52). If residual expression of BAF is sufficient to complement the PICs in a virus-infected cell, the results might downplay the function of BAF during retroviral infection. Thus, the use of VRKs in combination with the siRNA-based knockdown experiments would be of help in clarifying functional aspect of BAF during MoMLV and HIV-1 replication. In addition, our finding that certain kinases including VRKs are capable of disrupting PIC function could be the basis of a future therapeutic strategy for the treatment of retroviral infection.

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FOOTNOTES

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FIGURE LEGENDS

**Fig. 1.** Disruption of BAF/DNA complex by VRK1 in vitro. A, Activity of GST-fused murine VRK1 for BAF phosphorylation. BAF (500 nM) was incubated with 500 nM GST-VRK1 or GST in the presence of MgCl₂, cold ATP, and [γ-³²P]ATP. Proteins were resolved by SDS-PAGE and visualized by autoradiography (top panel). A parallel in vitro kinase assay was also performed in the absence of [γ-³²P]ATP and BAF was detected by immunoblotting analysis using anti-BAF monoclonal antibody (bottom panel). The black, gray, and white arrowheads indicate unphosphorylated, hypophosphorylated, and hyperphosphorylated forms of BAF, respectively. Masses of molecular weight standards are indicated at the left.

B, Gel mobility shift assay of BAF/DNA complex. Short substrate DNA (691-bp, lane 1) was incubated with BAF to form BAF/DNA complex (lane 2) and then treated with 0.2, 1, and 5 nM GST-VRK1 (lanes 3-5) or GST (lanes 6-8) in the presence of 1 mM ATP and 10 mM MgCl₂. Treatment of BAF/DNA complex with GST-VRK1 was also carried out without ATP (lane 9) or MgCl₂ (lane 10). Reaction samples were loaded on a 0.4% agarose gel in TAE buffer and substrate DNA was detected by Southern blotting analysis.

C, BAF release assay. BAF/DNA complex was formed with 500-bp DNA that had been conjugated with paramagnetic beads. Treatments of the BAF/DNA complex were performed with GST-VRK1 (lanes 3 and 4), a catalytic domain mutant of the VRK1 (GST-VRK1 D177A, lanes 7 and 8), and GST (lanes 9 and 10) in the presence of ATP. Treatment with GST-VRK1 was also carried out in the absence of ATP (lanes 5 and 6). After the treatment, DNA complex was washed using a magnetic stand and BAF in DNA bound (B) fractions (lanes 1, 3, 5, 7, and 9) and released (R) fractions (lanes 2, 4, 6, 8, and 10) was detected by immunoblotting analysis using anti-BAF monoclonal antibody. The black, gray, and white arrowheads indicate unphosphorylated, hypophosphorylated, and hyperphosphorylated forms of BAF, respectively. Masses of molecular weight standards are indicated at the left.

D, Velocity sedimentation analysis of BAF/DNA complex following VRK1 treatment. BAF was incubated with long substrate DNA (5,386-bp, panel 1) to form a nucleoprotein complex (panel 2) and subsequently treated with GST-VRK1 (panel 3) or GST (panel 4) in the presence of ATP and MgCl₂. The GST-VRK1 treatment was also performed in the absence of ATP (panel 5). Each reaction was loaded on a 15-30% sucrose gradient and centrifuged. The gradient was fractionated from the top to bottom into 10 fractions and substrate DNA in each fraction was detected by Southern blotting analysis.

**Fig. 2.** Dissociation of BAF from MoMLV PICs by VRK1. A, Immunoprecipitation analysis of the PIC with anti-BAF antibody following VRK1 treatment. Cytoplasmic extract from MoMLV-infected
cells was incubated with GST-VRK1 or GST in the presence of 1 µM ATP. Salt-stripped PICs were prepared by the treatment with 750 mM KCl (high-salt) and subsequent gel-filtration (10). Treated PICs were then subjected to immunoprecipitation assays (IP) using anti-BAF rabbit IgG or control IgG. Viral DNA was extracted from captured complexes and detected by Southern blotting for MoMLV LTR sequence. B, Sedimentation properties of PICs following VRK1 treatment. MoMLV PIC fraction treated with GST (panel 1), GST-VRK1 (panel 2), and high-salt (panel 3) were layered on 15-30% sucrose gradients and centrifuged. The gradient was fractionated from the top to bottom into 20 fractions and viral DNA in each fraction was detected by Southern blotting.

**Fig. 3.** Abolition of intermolecular integration activity of MoMLV PICs by VRK1. A, VRK1-mediated inhibition of PIC function. Prior to *in vitro* integration activity assay, PICs were treated with 20, 100, and 500 nM GST-VRK1 (lanes 2-4), GST (lanes 5-7), or GST-VRK1<sup>D177A</sup> (100 nM, lane 8) in presence of 1 µM ATP. PIC samples including salt-stripped PIC (lane 9) were then mixed with the integration reaction containing ΦX174 DNA as a target DNA. After incubation, DNA products from the reaction were digested with *Bam*HI and detected by Southern blotting for MoMLV LTR sequence. The 11.0-kb band results from intermolecular integration of the viral DNA into target ΦX174 DNA (inter). The 5.6-kb band and the smear below it result from autointegration of the viral DNA into itself (auto). The 3.7- and 1.9-kb bands are the unreacted viral DNA containing 5’ LTR (L-end) and 3’ LTR (R-end), respectively (7). The percentages of intermolecular integration activity (% inter) and autointegration activity (% auto) were measured by ImageGauge software. B, Effect of a kinase inhibitor on the VRK1-mediated disruption of the PIC function. PICs were pretreated with 500 nM GST-VRK1 and 100 µM staurosporine without addition of ATP.

**Fig. 4.** Inactivation of BAF function in PIC integration activity by VRK1-mediated phosphorylation. A, Preparation of hyperphosphorylated BAF used for *in vitro* integration activity assay. *In vitro* kinase assay was carried out using unphosphorylated BAF (black arrowhead) and GST-VRK1. Hyperphosphorylated BAF (P-BAF, white arrowhead) was then purified by a gel filtration column. Masses of molecular weight standards are indicated at the left. B, Activities of unphosphorylated and hyperphosphorylated forms of BAF in the function of the PIC. Initial PICs (lanes 1-5) and salt-stripped PICs (lanes 6-10) were incubated with either BAF or P-BAF, and subjected to *in vitro* integration activity assay.

**Fig. 5.** Comparative analyses of VRK family proteins in disruption of the PIC function. A, Schematic representation of GST-fused VRK1, VRK2, and VRK3 used in assay. The conserved catalytic domains are indicated by shaded boxes. NLS, nuclear localization signal. B, Kinase activities of VRK family proteins for BAF phosphorylation. *In vitro* kinase assay of BAF was performed with GST-fused VRKs in the presence of [γ-<sup>32</sup>P]ATP. After 2 h incubation, phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography (top panel). A parallel assay was also carried out using cold ATP and BAF was detected by immunoblotting analysis using anti-BAF monoclonal antibody (bottom panel). The black, gray, and white arrowheads indicate unphosphorylated, hypophosphorylated, and hyperphosphorylated forms of BAF, respectively. Masses of molecular weight standards are indicated at the left. C, Ability of the VRKs to disrupt BAF/DNA complex. Simple BAF/DNA complex was formed with paramagnetic beads-conjugated substrate DNA and treated with GST-VRK1 (lanes 1 and 2), GST-VRK2 (lanes 3 and 4), GST-VRK3 (lanes 5 and 6), or GST (lanes 7 and 8) for 1 min (top panel) and 60 min (bottom panel). BAF/DNA complex was washed using a magnetic stand and BAF in DNA bound (B) fractions (lanes 1, 3, 5, and 7) and released (R) fractions (lanes 2, 4, 6, and 8) were detected by immunoblotting analysis using anti-BAF monoclonal antibody. The black, gray, and white arrowheads indicate unphosphorylated, hypophosphorylated, and hyperphosphorylated forms of BAF, respectively. Masses of molecular weight standards are indicated at the left. D, Activities of VRK family proteins in inhibition of PIC function. MoMLV PICs were pretreated with 500 nM GST-VRK1 (lane 2), GST-VRK2 (lane 3), GST-VRK3 (lane 4), and GST (lane 5) in presence of 1 µM ATP, and then subjected to *in vitro*
integration activity assay. Inter, intermolecular integration; Auto, autointegration; L-end and R-end, unreacted viral DNA containing 5’ LTR and 3’ LTR, respectively (7).

**Fig. 6.** Involvement of endogenous VRK1 in disruption of the MoMLV PIC function. A, Abolishment of intermolecular integration activity by increasing concentration of additional ATP. PICs were pretreated with (+) or without (-) GST-VRK1 in the presence of additional 1 µM (lanes 1 and 2), 10 µM (lanes 3 and 4), 100 µM (lanes 5 and 6), and 1,000 µM (lanes 7 and 8) ATP, and subjected to *in vitro* integration activity assay. B, Effect of various nucleotide treatments in PIC integration activity. Pretreatments of PICs were carried out with 1 mM NTP (ATP, GTP, CTP, or UTP, lanes 2-5) and dNTP (dATP, dGTP, dCTP, dUTP, or dTTP, lanes 6-10). Then, the PICs were subjected to *in vitro* integration activity assay. C, Dissociation of BAF from the PICs following the nucleotide treatments. Immunoprecipitation analysis (IP) using anti-BAF IgG was performed with PICs that had been treated with 1 mM ATP, GTP, and dATP. As a control experiment, PICs were also treated with 1 mM CTP prior to the immunoprecipitation assay. Viral DNA in captured complexes was detected by Southern blotting. D, Depletion of endogenous VRK1 from MoMLV-infected cells. NIH3T3 cells were transfected with siRNA against VRK1 or nonspecific control siRNA twice and then infected with MoMLV for 7 h. Cytoplasmic extract containing the PICs was subjected to immunoblotting analysis using anti-VRK1 rabbit polyclonal IgG. Upper bands shown by asterisks are presumably larger splicing variants of murine VRK1 (31). MW, molecular weight marker. E, Effect of added ATP in intermolecular integration activity of the PICs from VRK1-knockdown cells. MoMLV PICs were isolated from mock- (lanes 1 and 2), VRK1 siRNA- (lanes 3 and 4), or control siRNA-transfected cells (lanes 5 and 6) and incubated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) 1 mM ATP. The PICs were subjected to *in vitro* integration activity assay.
FIGURE 1A and B

A

 Autoradiograph of $^{32}$P

| No protein | GST-VRK1 | GST |
|------------|----------|-----|
| 120        | 5        | 60  |
| 120        | 5        | 60  |
| 120        | 5        | 60  |

Immunoblotting with anti-BAF antibody

B

BAF/DNA complex incubated with

| Substrate DNA | No GST-VRK1 | GST-VRK1 | GST |
|---------------|-------------|----------|-----|
| 1             | 2           | 3        | 4   |
| 5             | 6           | 7        | 8   |
| 9             | 10          |          |     |

BAF/DNA complex

Substrate DNA
FIGURE 1C and D

(Suzuki et al.)
Figure 2

A

MoMLV PICs treated with

|       | No protein | GST-VRK1 | GST | High-salt |
|-------|------------|----------|-----|-----------|

IP: Anti-BAF IgG

Input PICs

IP: Control IgG

B

MoMLV PICs treated with

|                | Top | Fractions | Bottom |
|----------------|-----|-----------|--------|
|                | 1   | 2         | 3      |
| GST            |     |           |        |
| GST-VRK1       |     |           |        |
| High-salt      |     |           |        |
A  MoMLV PICs treated with

|                | No protein | GST-VRK1 | GST | GST-VRK1\_D177A | High-salt |
|----------------|------------|----------|-----|-----------------|-----------|
| nM             | 20         | 100      | 500 | 20              | 100       |

Lane number 1-9

% inter
14 5 4 2 17 12 15 10 <1

% auto
17 26 26 27 18 16 18 16 36

--- Inter (11.0-kb)
--- Auto (5.6-kb)
--- L-end (3.7-kb)
--- R-end (1.9-kb)

B  MoMLV PICs treated with

|                | No protein | GST-VRK1 | GST | Staurosporine |
|----------------|------------|----------|-----|---------------|
|                | -          | -        | -   | -             |
|                | +          | +        | +   | +             |

Lane number 1-5

% inter
18 5 17 22 22

% auto
8 20 13 10 10

--- Inter (11.0-kb)
--- Auto (5.6-kb)
--- L-end (3.7-kb)
--- R-end (1.9-kb)
FIGURE 4

(A) Initial PICs incubated with No protein, BAF, or P-BAF at concentrations of 10 and 100 nM. The gels show the following bands:
- Inter (11.0-kb)
- Auto (5.6-kb)
- L-end (3.7-kb)
- R-end (1.9-kb)

(B) Salt-stripped PICs incubated with No protein, BAF, or P-BAF at concentrations of 10 and 100 nM. The gels show the following bands:
- Inter (11.0-kb)
- Auto (5.6-kb)
- L-end (3.7-kb)
- R-end (1.9-kb)

| Lane number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-------------|---|---|---|---|---|---|---|---|---|----|
| % inter     | 9 | 11| 29| 8 | 7 | 2 | 5 | 28| 2  | 2  |
| % auto      | 39| 37| 24| 41| 42| 49| 39| 23| 50 | 44 |
A

GST-VRK1

1 37

Catalytic domain

266

356 360 396

GST

GST

NLS

GST-VRK2

1 29

264

503

GST

GST

GST-VRK3

1 49 63

145

374 454

GST

GST

NLS

B

Autoradiograph of $^{32}$P

Immunoblotting with anti-BAF antibody

[kDa]

No protein

GST-VRK1

GST-VRK2

GST-VRK3

GST

8.5

12

[kaDa]
FIGURE 5C and D

MoMLV PICs treated with

| No protein | GST-VRK1 | GST-VRK2 | GST-VRK3 | GST   |
|------------|----------|----------|----------|-------|
| B          | R        | B        | R        | B     |

Lane number

% inter

20  4  11  24  18

% auto

27  57  35  16  25

1 min treatment

60 min treatment

[Table of band intensities for different treatments and time points]

[Schematic diagram showing band patterns for different treatments and time points]
FIGURE 6A and B

A

MoMLV PICs treated with

| ATP [μM] | 1   | 10  | 100 | 1000 |
|----------|-----|-----|-----|------|
| GST-VRK1 | -   | +   | -   | +    |

- Inter (11.0-kb)
- Auto (5.6-kb)
- L-end (3.7-kb)
- R-end (1.9-kb)

Lane number

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|
| % inter | 23 | 3 | 29 | 3 | 6 | 4 | 5 | 4 |
| % auto   | 11 | 30 | 16 | 39 | 40 | 41 | 32 | 35 |

B

MoMLV PICs treated with

| No NTP/dNTP addition | NTP | dNTP |
|-----------------------|-----|------|
| A | G | C | U | A | G | C | U | T |

- Inter (11.0-kb)
- Auto (5.6-kb)
- L-end (3.7-kb)
- R-end (1.9-kb)

Lane number

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|-----|
| % inter | 28 | 2 | 21 | 45 | 46 | <1 | 47 | 43 | 29 |
| % auto   | 7 | 39 | 33 | 10 | 12 | 39 | 14 | 11 | 9 | 11 |
FIGURE 6C

MoMLV PICs treated with

| No NTP/dNTP addition | ATP | GTP | dATP | CTP |
|----------------------|-----|-----|------|-----|

IP: Anti-BAF IgG

Input PICs

IP: Control IgG
FIGURE 6D and E

D

![Western blot with protein bands labeled (kDa) and VRK1 as indicated by an arrow.](chart)

E

| ATP   | MoMLV PICs from | Mock transfected cell | siVRK1 transfected cell | siControl transfected cell |
|-------|----------------|-----------------------|-------------------------|---------------------------|
| -     | 1              | Inter (11.0-kb)       | Auto (5.6-kb)           | L-end (3.7-kb)            |
| +     | 2              |                       |                         |                           |
| -     | 3              | Inter (11.0-kb)       | Auto (5.6-kb)           | L-end (3.7-kb)            |
| +     | 4              |                       |                         |                           |
| -     | 5              |                       |                         | R-end (1.9-kb)            |
| +     | 6              |                       |                         |                           |

(Suzuki et al.)
Functional disruption of the moloney murine leukemia virus preintegration complex by vaccinia-related kinases
Yasutsugu Suzuki, Kanako Ogawa, Yoshio Koyanagi and Youichi Suzuki

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