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Rep68 protein of adeno-associated virus type 2 interacts with 14-3-3 proteins depending on phosphorylation at serine 535

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Abstract

Rep78/68 proteins of adeno-associated virus type 2 (AAV-2) are involved in many aspects of the viral life cycle, including replication, gene expression, and site-specific integration. To understand the molecular mechanisms of the actions of Rep proteins, we searched for Rep68-interacting cellular proteins by utilizing a one-step affinity purification technique and identified two members of 14-3-3 proteins (14-3-3 ε and γ). We found that phosphorylation of ^{535}Ser at the carboxy terminus of Rep68 was critical for its association with 14-3-3. The association of 14-3-3 proteins to Rep68 resulted in reduction of the affinity of Rep68 for DNA. Furthermore, genome DNA replication of a recombinant mutant virus carrying a phosphorylation-deficient Rep68 (Ser535Ala) was more efficient than that of the wild-type virus. These results suggest that phosphorylation of Rep68 and subsequent association with 14-3-3 proteins regulates Rep-mediated functions during the AAV life cycle.

Keywords: AAV; Rep; 14-3-3; Phosphorylation; DNA binding; Replication

Introduction

Adeno-associated virus type 2 (AAV-2) is a nonpathogenic human parvovirus. For efficient AAV DNA replication and gene expression, co-infection of a helper virus such as an adenovirus or a herpesvirus is required. In the absence of a helper virus, AAV establishes latent infection by integrating the viral genome into a specific site on chromosome 19 (*AAVS1*); although, a low level of helper-independent AAV replication does take place in cells exposed to genotoxic stress (Leonard and Berns, 1994; Muzyczka, 1992).

AAV contains a linear single-stranded DNA genome of approximately 4.7 kb flanked by palindromic inverted terminal repeats (ITRs), which serve as the viral origin of replication. The AAV genome consists of two major open reading frames, *rep* and *cap*. The *cap* gene encodes three viral structural proteins: VP1, VP2, and VP3. The *rep* gene encodes four nonstructural proteins: Rep78, Rep68, Rep52, and Rep40, which are produced by alternative promoter utilization and differential splicing (Leonard and Berns, 1994; Muzyczka, 1992).

Rep78 and its C-terminal spliced version, Rep68, regulate many aspects of the viral life cycle, including DNA replication (Ni et al., 1998; Wang and Srivastava, 1998), gene expression (Horer et al., 1995; Kyostio et al., 1994; Pereira et al., 1997), and site-specific integration (Linden et al., 1996a,1996b; Samulski et al., 1991; Weitzman et al., 1994; Young et al., 2000). They are multifunctional proteins with various activities, including sequence-specific DNA binding (McCarty et al., 1994a,1994b; Ryan et al., 1996), site- and strand-specific endonuclease (Brister and Muzyczka, 2000; Walker et al., 1997a), helicase (Walker et al., 1997b), and ATPase activities (Im and Muzyczka, 1990, 1992; Wonderling et al., 1995; Wu et al., 1999; Zhou

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et al., 1999). Rep52 and Rep40, N-terminal truncated forms of Rep78 and Rep68, respectively, do not exert sequence-specific DNA binding but have been shown to have ATP-dependent helicase activities and to be involved in the accumulation and encapsidation of single-stranded genomes (Chejanovsky and Carter, 1989; King et al., 2001).

AAV has also been shown to have antiproliferative effects on various types of cells. In many cases, this property has been mapped to the larger Rep proteins, and they have been shown to inhibit transformation by viral and cellular oncogenes, viral and cellular DNA synthesis, and transcription from a variety of promoters (Batchu et al., 2001; Hermanns et al., 1997; Kube et al., 1997; Saudan et al., 2000).

Collectively, the Rep proteins of AAV are pleiotropic effectors of the viral life cycle and cellular events. It has been shown that Rep proteins functionally interact with host cellular proteins (Costello et al., 1997; Di Pasquale and Stacey, 1998; Pereira and Muzyczka, 1997; Weger et al., 1999). In this study, we searched for cellular proteins that interact with the Rep68 protein. We employed latex beads onto which various biologically active components, such as chemical compounds (Shimizu et al., 2000), nucleic acids (Handa, 1992; Wada et al., 1996), and proteins (Hatakeyama et al., 1997), can be covalently immobilized. We immobilized the Rep68 protein onto latex beads and, through a one-step affinity chromatography, purified two proteins from HeLa cell nuclear extracts identified as members of the 14-3-3 protein family. We found that 14-3-3 proteins specifically associate with Rep68 but not with Rep78, and that this association is dependent on phosphorylation of serine 535 at the carboxy terminus of Rep68. The association of 14-3-3 reduced the affinity of Rep68 for ITR and *AAVS1*. A mutant virus in which ^{535}Ser in Rep68 is changed to Ala showed a higher level of viral DNA replication than wild type, which is likely due to the lack of phosphorylation at position 535 of the mutant Rep68 and its subsequent inability to associate with 14-3-3 proteins. Taken together, these results suggest that the activity of AAV Rep68 is regulated through phosphorylation and interaction with 14-3-3 proteins.

Results

Identification of members of 14-3-3 proteins as Rep68 interactors by affinity chromatography using Rep68-immobilized latex beads

To search for factors interacting with Rep68 protein, we utilized a one-step affinity purification procedure involving high-performance latex beads onto which recombinant Rep68 protein was immobilized. We produced recombinant Hisx6-tagged Rep68 protein (His-Rep68) in insect cells and purified the protein by nickel column chromatography to near homogeneity (data not shown). We then immobilized the His-Rep68 protein onto latex beads. The amino, thiol, and imidazole groups of the polypeptides are expected to directly react with the carbon atoms on the tosyl group at the end of the spacer arms of the SG-N-EOTs beads (Fig. 1A).

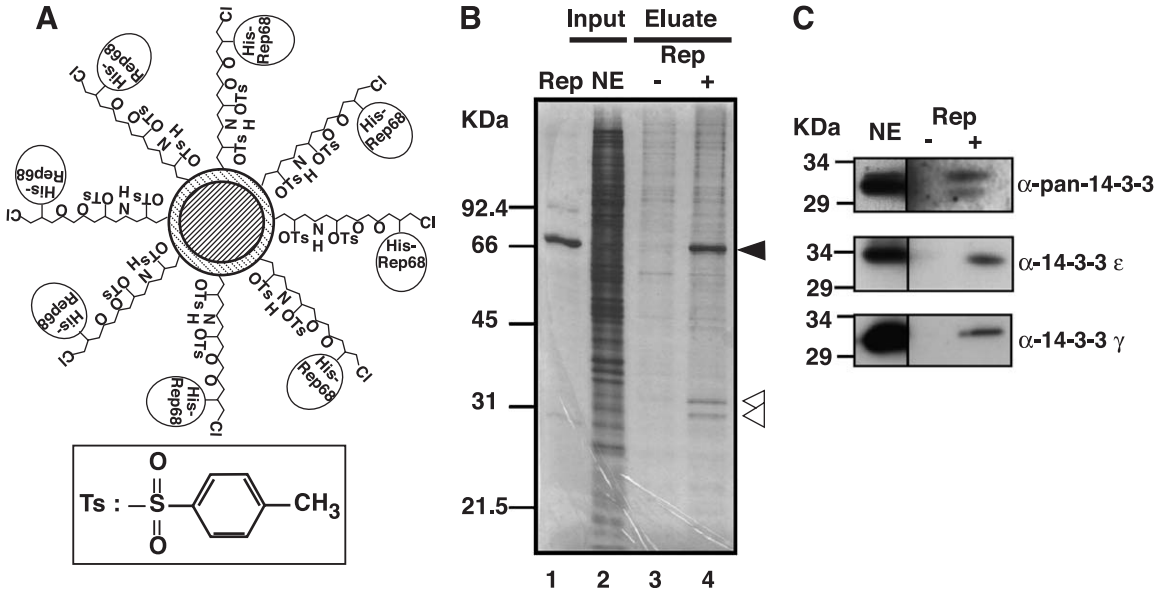

Fig. 1. Identification of Rep68 interactors as members of the 14-3-3 family of proteins. (A) Schematic representation of Rep68-immobilized latex beads. Tosyl-activated latex beads (SG-N-EOTS) were coupled with His-Rep68. Ts of inset shows a tosyl group of the latex beads. (B) Affinity purification using His-Rep68-immobilized latex beads. Purified recombinant His-Rep68 protein (lane 1). Nuclear extracts of HeLa cells (lane 2). Purified fractions of control (lane 3) and His-Rep68-immobilized latex beads (lane 4). Filled and open arrows show His-Rep68 and Rep-interacting cellular proteins, respectively. (C) Immunoblotting. Nuclear extracts (NE) and the eluted fraction from beads alone (Rep−) and His-Rep68-immobilized beads (Rep+) were immunoblotted with anti-pan-14-3-3 (top), anti-14-3-3 ε (middle), and anti-14-3-3 γ (bottom) antibodies.

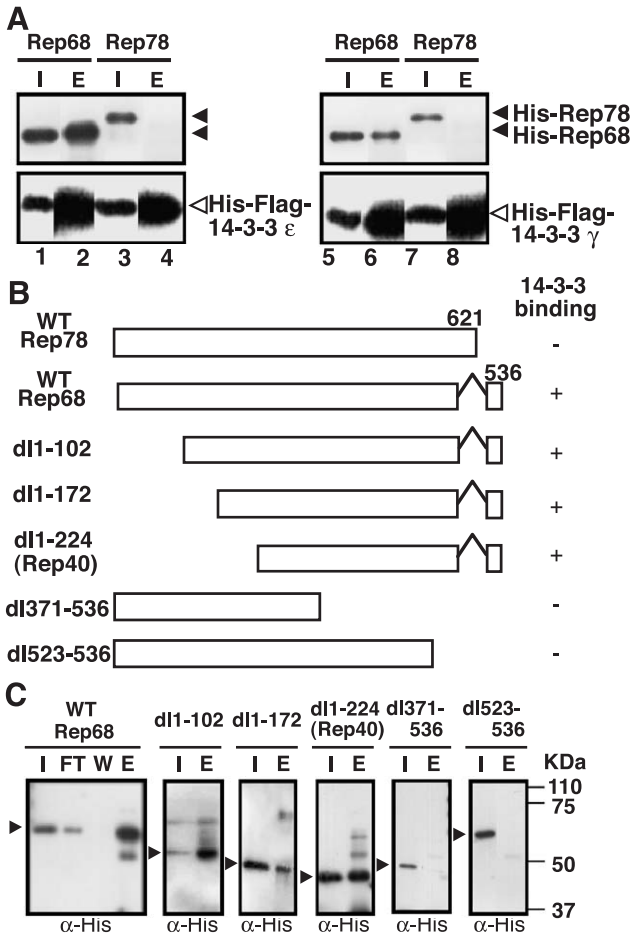

Fig. 2. Determination of the 14-3-3-binding domain within the Rep protein. (A) Specific interaction of 14-3-3 with the Rep68 but not with the Rep78 protein. Purified recombinant His-Rep68 (2 μg) and His-Rep78 (1 μg) proteins expressed in insect cells were incubated with bacterially expressed and purified His-Flag-14-3-3 ε (1 μg) or His-Flag-14-3-3 γ (1 μg), and subjected to Flag affinity resin precipitation. Ten percent input (I) and elution (E) fractions were separated by SDS-PAGE and were immunoblotted with anti-His (top) and anti-Flag (bottom) antibodies. (B) Schematic representation of His-Rep proteins tested for binding to 14-3-3. The coding regions are represented by boxes, and introns are shown as carats. Their abilities to interact with 14-3-3 proteins are also summarized on the right. (C) Co-precipitation assay in vitro. Wild-type and deletion mutants of His-Rep68 (2 μg) were expressed in insect cells, purified, and tested for co-precipitation with His-Flag-14-3-3 γ (1 μg). Ten percent input (I), 10% flow-through (FT), 10% wash (W), and elution fractions (E) were analyzed by SDS-PAGE and were immunoblotted with anti-His antibody. Filled arrows indicate Rep proteins.

Our previous findings showed that the imidazole groups of tagged histidine residues (Hisx6) were highly reactive, and His-tagged proteins could be immobilized onto the SG-N-EOTs beads efficiently, even at 4°C (Hatakeyama et al., 1997). Using this system, we could immobilize approximately 20 μg of the recombinant His-Rep68 protein onto 1 mg of the latex beads (data not shown).

The His-Rep68-carrying beads were incubated with HeLa cell nuclear extracts, and proteins specifically bound to the beads were eluted by a high-salt buffer and analyzed by SDS-PAGE. As shown in Fig. 1B, two proteins of approximately 32 and 30 kDa in size were eluted from the His-Rep68-carrying beads (lane 4, open arrowheads), but not from the control beads (lane 3). In lane 4, His-Rep68 (68 kDa) was also found in the eluate (filled arrowhead), as revealed by immunoblotting using an anti-Rep antibody (data not shown). These were probably derived from non-covalently associated His-Rep68 protein on the beads, because Rep68 forms multimeric complexes (Smith et al., 1997).

The 32- and 30-kDa Rep binding proteins were identified as the 14-3-3 isoform ε and 14-3-3 isoform γ, respectively, by tryptic digestion and microsequencing of HPLC-fractionated peptides (data not shown). The identities of the purified proteins were confirmed by immunoblot analyses. As shown in Fig. 1C, both the 32- and 30-

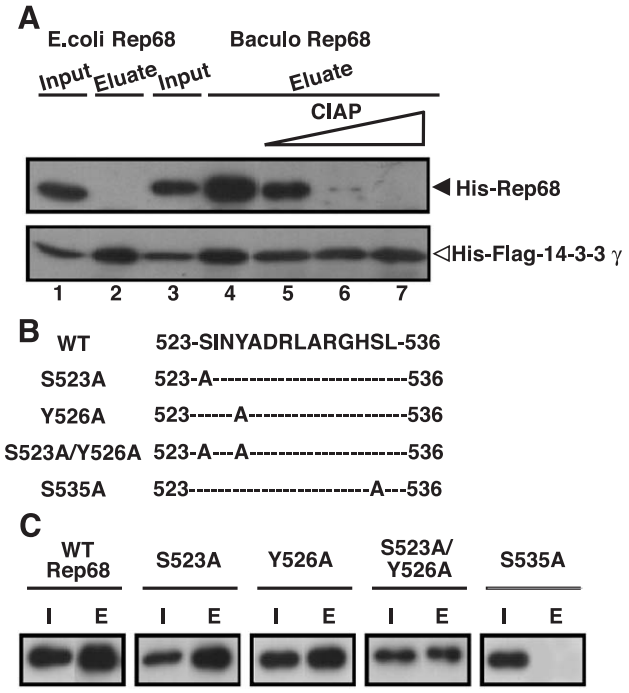

Fig. 3. [535]Ser in the C-terminal region of Rep68 is critical for interaction with 14-3-3 proteins. (A) Phosphorylation-dependent interaction of Rep68 and 14-3-3. Purified His-Rep68 protein (2 μg) expressed in *E. coli* (lanes 1 and 2) or in insect cells (lanes 3–7) were incubated with His-Flag-14-3-3 γ (1 μg) and subjected to Flag affinity resin purification. Before incubation with 14-3-3 γ, Rep68 proteins expressed in insect cells were untreated (lane 4) or treated with 0.02, 0.2, and 2 units of alkaline phosphatase (CIAP) (lanes 5–7). Input and elution fractions were then immunoblotted by anti-Rep (top) or anti-Flag (bottom) antibodies. (B) Amino acid sequence of the C-terminal region of Rep68 protein. Wild-type and each substitution mutants with serine and/or tyrosine residues substituted to alanine are indicated. (C) Interaction of Rep68 substitution mutants with 14-3-3 in vitro. Purified wild-type and mutant His-Rep68 proteins (2 μg) expressed in insect cells were tested for interaction with His-Flag-14-3-3 γ (1 μg). Ten percent input (I) and elution (E) fractions separated by SDS-PAGE were immunoblotted with anti-Rep antibody.

kDa proteins reacted with a polyclonal anti-pan-14-3-3 antibody (H8, broadly reactive with the 14-3-3 protein family members: top panel). The 32- and 30-kDa bands were found reactive to the antibodies specific to 14-3-3 ε (T-16: middle panel) and 14-3-3 γ (C-16: bottom panel), respectively.

14-3-3 Proteins interact with Rep68, but not with Rep78, in vitro

To demonstrate a direct interaction between Rep proteins and 14-3-3 proteins, Hisx6-Flag dual tagged 14-3-3 ε and 14-3-3 γ fusion proteins (His-Flag-14-3-3 ε and His-Flag-14-3-3 γ) were expressed in *Escherichia coli* and used for in vitro binding assay. In addition to His-Rep68, we also produced the other larger Rep protein, Rep78 (His-Rep78), in insect cells to examine its interaction with 14-3-3 proteins. Each fusion protein was first purified by nickel column chromatography through their Histidine-tag. Then His-Rep68 or His-Rep78 was mixed with either His-Flag 14-3-3 ε or γ and co-immunoprecipitation was carried out using anti-Flag antibody affinity resin. The precipitates were analyzed by immunoblotting with anti-His-Tag antibody to detect His-tagged proteins, that is, His-Flag 14-3-3 ε and γ, His-Rep68, and His-Rep78. As shown in Fig. 2A, His-Rep68 protein co-precipitated with His-Flag-14-3-3 ε (lane 2) and γ (lane 6), whereas His-Rep78 did not (lanes 4 and 8). The results indicate that Rep68 protein, but not Rep78

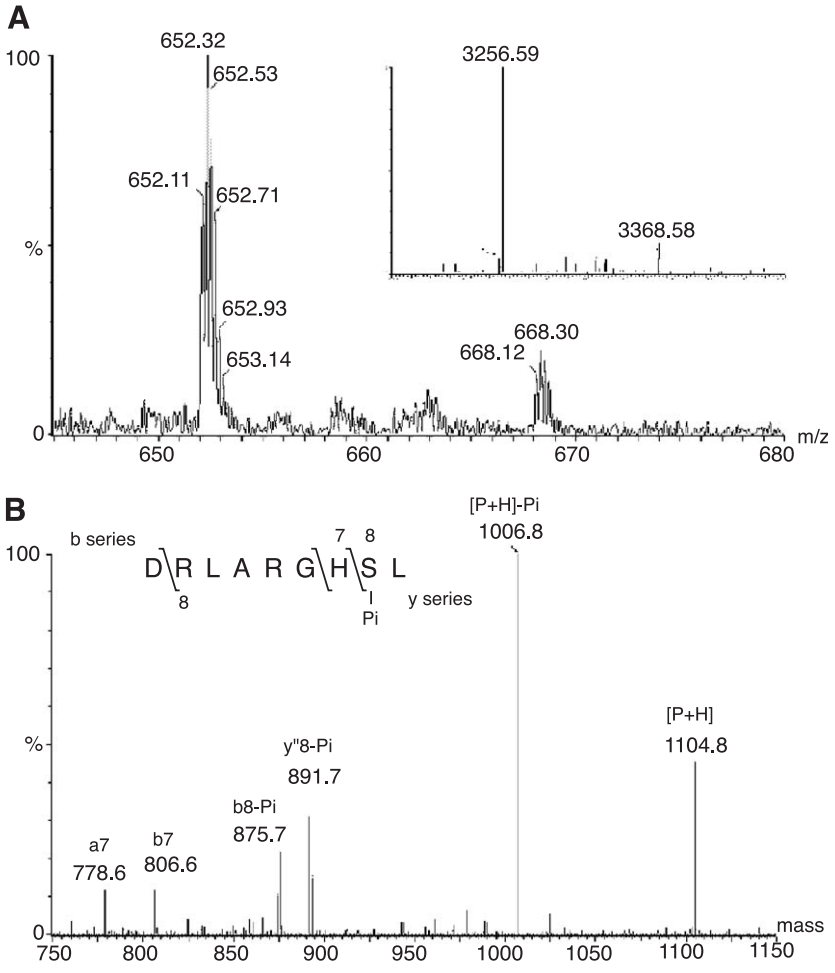

Fig. 4. Localization of the Rep68 phosphorylation site at ^{535}Ser by mass spectrometry. (A) Recombinant His-Rep68 protein was digested with Lys-C endopeptidase and analyzed by the mass spectrometer connected directly to a capillary HPLC system (LC/MS). A peak corresponding to a molecular mass (Mr.) of 652.11 was assigned to the number of electrostatic charge unit $z = 5$ of the C-terminal peptide RVRESVAQPSTSDAEASINYADRLARGHSL, and another peak shows a shift corresponding to 1 mol of phosphate (Mr. 668.12). The inset shows unmodified and phosphorylated peptides of its parent ion, 3256.59 and 3336.58, respectively. (B) His-Rep68 protein was digested with Asp-N endopeptidase and analyzed by mass spectrometry. A Mr. 1104.8[P + H] phosphopeptide candidate was identified and subsequently sequenced by mass spectrometry/mass spectrometry (MS/MS). The resulting mass spectrum was assigned to the C-terminal peptide DRLARGHSL (inset). N-terminal and C-terminal peptide sequence ions by cleavage of the amide bonds are indicated with b and y series, respectively, and their calculated masses are shown adjacent to the amino acid sequence. a7, seventh N-terminal peptide sequence ions by cleavage of C-Cα bond, were detected. Additionally, the ion signals corresponding to peptide fragments that have lost the phosphate group H_3PO_4 by β-elimination (indicted with -Pi) were detected.

protein, directly associates with both 14-3-3 ε and 14-3-3 γ proteins.

Rep68 interacts with 14-3-3 proteins through its C-terminal region

To identify the Rep68 domain involved in interactions with 14-3-3 proteins, a series of truncated His-Rep fusion proteins were expressed in insect cells (Fig. 2B), and their ability to bind 14-3-3 proteins was examined. The purified His-tagged Rep68 fragments were incubated with His-Flag-14-3-3 γ, and co-immunoprecipitation with anti-Flag antibody resin was carried out, followed by immunoblotting with anti-His (Fig. 2C). All the N-terminally deleted mutants (dl 1–102, dl 1–172) and the naturally occurring smaller Rep40 protein could bind to His-Flag 14-3-3 γ, whereas C-terminal truncated mutants (dl 371–536, dl 523–536) could not. Similar results were obtained using 14-3-3 ε instead of γ (data not shown). As mentioned above, Rep68 is a C-terminally spliced version of Rep78 in which amino acids 530–621 are absent and are replaced by the amino acid sequence LARGHSL. Thus, our results suggest that these unique C-terminal residues of Rep68 (523–536), which are also found in Rep40 but not in Rep78/52, are necessary for interaction between Rep68 and 14-3-3 proteins.

^{535}Ser in the C terminus of Rep68 is critical for interaction with 14-3-3 proteins

In contrast to the results using His-Rep68 expressed in insect cells, our attempt to co-precipitate His-Rep68 protein produced in *E. coli* with recombinant 14-3-3 proteins was unsuccessful (Fig. 3A, lanes 1 and 2). Because 14-3-3 proteins bind preferentially to phosphorylated proteins (Fu et al., 2000; Yaffe et al., 1997), we tested whether the interaction of Rep68 with 14-3-3 proteins is dependent upon phosphorylation of Rep68. As shown in Fig. 3A (lanes 3–7), when insect cell-derived His-Rep68 protein was treated with an increasing amount of alkaline phosphatase, its association with 14-3-3 proteins was abrogated. Similar results were obtained using 14-3-3 ε (data not shown). These results suggest that the Rep68 protein expressed in insect cells is phosphorylated and that phosphorylation is essential for interaction with 14-3-3 proteins.

To investigate the importance of phosphorylation of Rep68 in its association with 14-3-3 proteins, we generated a full-length mutant Rep68 protein in which ^{523}Ser and/or ^{526}Tyr or ^{535}Ser in the C-terminal region (amino acids 523–536) was substituted by Ala (Fig. 3B). These were expressed in insect cells, purified, and subjected to co-precipitation assays using His-Flag 14-3-3 proteins. Among these mutant Rep68 proteins, only the S535A mutant lacked the ability to interact with 14-3-3 γ (Fig. 3C) and with 14-3-3 ε (data not shown). These results indicate that phosphorylation at ^{535}Ser of Rep68 is critical for interaction with 14-3-3 proteins.

^{535}Ser of Rep68 is phosphorylated

As the results above suggest that the ^{535}Ser residue of the C-terminal region of Rep68 is most likely phosphorylated, we investigated the site of phosphorylation of His-Rep68 protein expressed in insect cells using a multidimensional mass spectrometer. His-Rep68 protein was digested with Lys-C endopeptidase and the fragments were resolved with a mass spectrometer directly connected to a capillary HPLC system (LC/MS). As shown in Fig. 4A, unphosphorylated and phosphorylated peptides corresponding to the C-terminal region of Rep68 were detected. The mass value of the major peak observed at *m*/*z* 652.11 (the number of electrostatic charge units *z* = 5) was in complete agreement with the calculated mass value for the unphosphorylated peptide fragment RVRESVAQPSTSDAEASINYADRLARGHSL of His-Rep68. The mass value of the minor peak (*m*/*z* 668.12, *z* = 5) was close to the value of the monophosphopeptide of the C-terminal region (calculated value *m*/*z* 668.13, *z* = 5). This indicates that the C-terminal region of Rep68 is partially phosphorylated at a single site.

To identify the phosphorylated amino acid residue in the C-terminal region, His-Rep68 was digested using a different Asp-N endopeptidase. The mass signal corresponding to the C-terminal peptide DRLARGHSL with mono-phosphorylation (*m*/*z* 1104.8, *z* = 1) was detected. This C-terminal peptide fragment contains only one potential phosphorylation site (^{535}Ser). Moreover, the phosphorylated peptide was analyzed with mass spectrometry/mass spectrometry (MS/MS) (Fig. 4B). A fragment ion (*m*/*z* 1006.8, *z* = 1) notably observed in the MS/MS spectrum was likely formed by the loss of H_3PO_4 from the parent ion. These results indicate that the phosphorylation site positioned near the C terminus

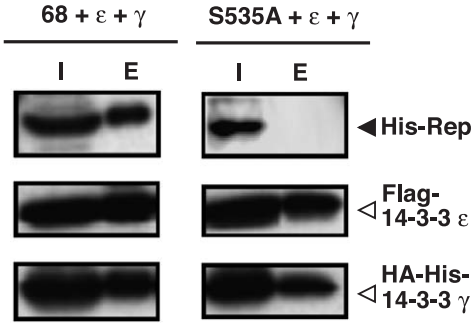

Fig. 5. Interaction of Rep and 14-3-3 proteins in vivo. Ternary complex formation of Rep68, 14-3-3 ε, and 14-3-3 γ. Baculoviruses expressing wild-type His-Rep68 or the S535A mutant and those expressing Flag-14-3-3 ε and HA-His-14-3-3 γ were co-infected to insect cells and purified by Flag affinity resin. Ten percent input (I) and elution (E) fractions were analyzed by immunoblotting with anti-Flag (Flag-14-3-3 ε) and anti-His (HA-His-14-3-3 γ and His-Rep) antibodies. 68, ε, γ, and S535A represent His-Rep68, Flag-14-3-3 ε, HA-His-14-3-3 γ, and the S535A mutant, respectively.

was most likely to be ^{535}Ser. Sequencing of the Lys-C endopeptidase-digested C-terminal peptide also yielded similar results (data not shown). These data clearly indicated that ^{535}Ser of Rep68 is phosphorylated.

Rep68 and 14-3-3 form ternary complexes in vivo

Previous reports have shown that 14-3-3 proteins form homo- and hetero-dimers (Fu et al., 2000; Jones et al., 1995). We therefore examined whether 14-3-3 ε and 14-3-3 γ form ternary complexes with Rep68. For this purpose, we expressed Flag-14-3-3 ε and HA-His-14-3-3 γ together with His-Rep68 or the His-tagged S535A mutant in insect cells using co-infection of baculoviruses directed to express these proteins. After precipitation of Flag-14-3-3 ε by anti-Flag affinity resin, the eluate was tested by immunoblotting using anti-His antibody to detect His-tagged 14-3-3 γ, Rep68, and S535A proteins. As shown in Fig. 5, wild-type His-Rep68 and HA-His-14-3-3 γ proteins were co-purified with Flag-14-3-3 ε, indicating that they form a ternary complex in insect cells. When ^{535}Ser of the His-Rep68 protein was mutated to Ala, the mutant His-Rep68 did not participate to the complex formation with Flag-14-3-3 ε and HA-His-14-3-3 γ. These results indicate that Rep68 and 14-3-3 form ternary complexes, and suggest that such ternary complexes could be formed in vivo.

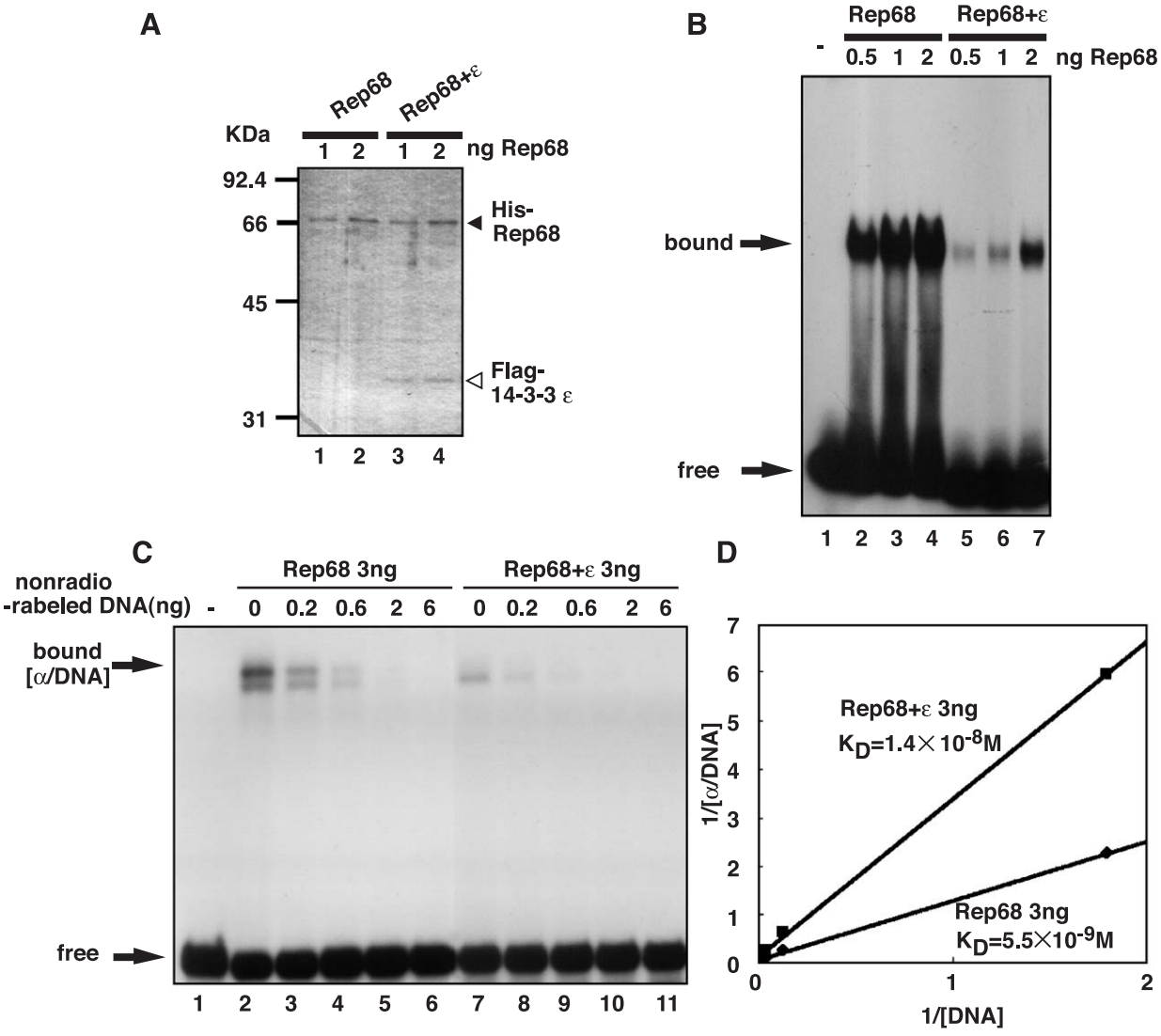

Fig. 6. DNA-binding activities of Rep68 alone and Rep68/14-3-3 ε complex. (A) Co-purification of Rep68 and 14-3-3 ε. Recombinant His-Rep68 (lanes 1 and 2) or His-Rep68/Flag-14-3-3 ε complex (lanes 3 and 4) were purified from insect cells, and aliquots were analyzed by SDS-PAGE and silver staining. (B) Gel mobility shift assay. The purified His-Rep68 (lanes 2–4) and His-Rep68/Flag-14-3-3 ε complex (lanes 5–7) were analyzed by gel mobility shift assay using ^{32}P-labeled ITR probe. The amounts of Rep68 protein used are indicated for both protein preparations. (C) Competitive gel mobility shift analysis. The indicated amounts of nonradiolabeled *AAVS1* were included in each reaction containing 3 ng His-Rep68 protein (lanes 2–6), or 3 ng His-Rep68 protein and 1.5 ng of Flag-14-3-3 ε (lanes 7–11), and 0.02 ng of ^{32}P-labeled *AAVS1* probe. (D) Lineweaver–Burk plot of the gel-shift assay shown in C. The radioactivity in each band corresponding to both free DNA and DNA–Rep68 complex was measured by STORM and plotted, and the calculated values of K_D were indicated.

The binding of 14-3-3 proteins to Rep68 reduces the affinity of Rep68 for DNA

To evaluate the functional significance of the association between 14-3-3 proteins and Rep68, we asked whether the DNA-binding activity of Rep68 could be modified upon its binding to 14-3-3 proteins. We purified His-Rep68 alone or His-Rep68 in complex with Flag-14-3-3 ε co-expressed in insect cells (Fig. 6A). To avoid inclusion of Flag-14-3-3 ε-unbound His-Rep68 fraction into the His-Rep68/Flag-14-3-3 ε complex, we sequentially purified the complex with anti-His Tag and anti-Flag antibodies. Then gel mobility shift analysis was carried out using ^{32}P-labeled hairpin ITR DNA as a probe with increasing amount of the purified His-Rep68 or His-Rep68/Flag-14-3-3 ε complex. The mobility patterns of the DNA–protein complexes were similar between His-Rep68 alone (Fig. 6B, lanes 2–4) and His-Rep68/Flag-14-3-3 ε (Fig. 6B, lanes 5–7), both having a single shifted band that increased in intensity with increasing amount of added proteins. However, at equivalent amounts of His-Rep68, much less DNA–protein complexes were formed in the His-Rep68/Flag-14-3-3 ε–DNA reaction.

To further analyze these differences quantitatively, an apparent dissociation rate of His-Rep68 alone and His-Rep68/Flag-14-3-3 ε from DNA were calculated by measuring the radioactivity of protein-bound and -unbound probes in the presence of various amount of unlabeled competitor DNA (Figs. 6C and D). We used the oligonucleotides *AAVS1* DNA as a probe for efficient competition. The gel-shift assay of the Rep68 and *AAVS1* DNA showed two bands with different intensity (Fig. 6C, lanes 2–4) that seemed to be derived from different multimers of Rep proteins bound to the probe. When higher amount of Rep68 was added to the reaction, six mobility shifted bands could be detected (data not shown). The similar results showing five to six bands upon gel-shift assay were also observed by others (Costello et al., 1997; McCarty et al., 1994b). Only the single band with much lower intensity was visible in His-Rep68/Flag-14-3-3 ε–DNA reaction (Fig. 6C, lanes 7 and 8), and presence of the competitor DNA mostly abolished the signal (Fig. 6C, lanes 3–6, 8–11). Fig. 6D shows the Lineweaver–Burk plot of the results shown in Fig. 6C. Equilibrium constant (K_D) of His-Rep68–DNA interaction and His-Rep68/Flag-14-3-3 ε–DNA interaction were 5.5×10^{-9} and 1.4×10^{-8} M, respectively, and His-Rep68/Flag-14-3-3 ε had about 2.5-fold lower affinity to *AAVS1* DNA than His-Rep68 alone. Two groups reported the apparent binding constant of Rep68 to the hairpin ITR, 9×10^{-10} M (Chiorini et al., 1994) and 6.3×10^{-9} M (McCarty et al., 1994b) and to its truncated linear ITR lacking secondary structures, 8×10^{-10} M (Chiorini et al., 1994) and 2×10^{-8} M (McCarty et al., 1994b). The affinity to Rep68 to our 47-bp *AAVS1* DNA fragment appears comparable to that seen in the previous studies. Our results indicate that the binding of Rep68 to DNA was lowered by association with 14-3-3 proteins.

Mutation of Rep68 ^{535}Ser to Ala increased AAV DNA replication

As the first step toward understanding the role of ^{535}Ser phosphorylation of Rep68 and the association between Rep68 and 14-3-3 proteins in vivo, we examined the level of DNA replication of wild-type AAV (AAV-WT) and a mutant AAV having a S535A substitution in the Rep68 protein (AAV-S535A). Cells were transfected with either AAV-WT or AAV-S535A DNA, followed by infection with adenovirus type 5. Then, low molecular weight DNA was isolated and the AAV genomic DNA was detected by Southern blotting and quantitative-PCR (Fig. 7). By Southern blot analysis, double-stranded dimer replicative form (RF) (D), monomer RF (M), and progeny single-stranded DNA (S) were detected in both AAV-WT-transfected and AAV-S535A-transfected cells (Fig. 7A). Interestingly, the level of DNA replication of AAV-WT was less than that of AAV-S535A irrespective of their replicative forms. The experiment was repeated six times and a higher level of DNA replication was consistently observed in the mutant AAV. When the amount of replicated AAV DNA was measured by quantitative-PCR, the amount of mutant AAV DNA was 3.1-fold over that of the wild-type (Fig. 7B). Thus, our results suggest that the association of 14-3-3 proteins to Rep68 negatively regulates AAV DNA replica-

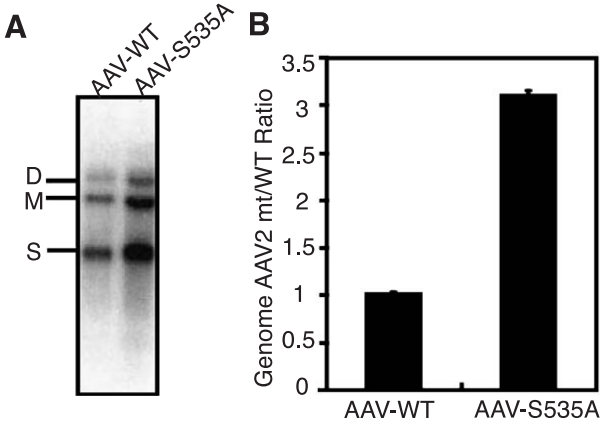

Fig. 7. Replication of AAV-WT DNA and AAV-S535A DNA. (A) HeLa cells were transfected either with wild-type AAV (AAV-WT) DNA or a mutant AAV DNA having S535A substitution in the Rep68 gene (AAV-S535A), and then infected with Ad5 at MOI of 10. The viral DNA of AAV-WT and AAV-S535A were extracted at 72 h post-infection, and analyzed by Southern blotting with ^{32}P-labeled AAV DNA as a probe. AAV DNA species are indicated: D, dimer replicative form (RF); M, monomer RF; S, single strands. (B) Quantitative-PCR analysis to measure extent of DNA replication. The viral DNA in AAV-WT and AAV-S535A samples were quantified using quantitative PCR method by comparing with known amount of purified AAV genome as the DNA standards. The each sample value is shown as the relative value to that of AAV-WT, which was set to 1. Data shown are the means ± SD of three experiments.

tion, and that such control may involve the reduction in DNA binding activity of Rep68 upon its association with 14-3-3 proteins. Mutating ^{535}Ser of Rep68 to Ala prevents binding of Rep68 to 14-3-3 proteins, and thus allows increased levels of AAV DNA replication in the mutant virus.

Discussion

In the present study, we employed a novel affinity purification system involving latex beads onto which recombinant Rep68 was attached and purified two factors from HeLa cell nuclear extracts that interact with AAV Rep68. The factors were identified as members of the 14-3-3 family of proteins (14-3-3 ε and 14-3-3 γ). The 14-3-3 proteins constitute a family of homo- and heterodimeric molecules and are highly conserved throughout eukaryotes. In mammals, at least seven distinct isoforms (β, δ, ε, γ, θ, σ, and ζ) have been identified to date (Fu et al., 2000; van Hemert et al., 2001). Despite the fact that several other isoforms are expressed in HeLa cells, we only obtained ε and γ isoforms in our affinity-purified fractions. It remains to be determined whether Rep68 also interacts with the other isoforms. The 14-3-3 proteins are present in relatively high abundance in cells and have been shown to associate with multiple cellular proteins having various functions. To date, more than 100 proteins, including several viral proteins, have been identified as 14-3-3-associated proteins. By binding to these target proteins, 14-3-3 proteins modify their activities and/or subcellular localization, and therefore numerous functions, such as mitogenesis, cell cycle control, apoptosis, and signal transduction, have been ascribed to 14-3-3 proteins (Fu et al., 2000; van Hemert et al., 2001).

The interaction between 14-3-3 proteins and their targets often requires phosphorylation of a serine residue, and phosphoserine-containing consensus motifs in 14-3-3 target proteins have been identified (Yaffe et al., 1997). Previous studies have demonstrated that all four Rep proteins are phosphorylated at serine residue(s) in AAV-infected and adenovirus co-infected cell cultures; however, the phosphorylated residue(s) have not yet been identified (Collaco et al., 1997). Our finding that phosphatase treatment of Rep68 abolished the binding of Rep68 to 14-3-3 proteins suggests that phosphorylation of Rep68 regulates the binding. Although there are no known consensus 14-3-3 binding motifs in the Rep protein, the unique C-terminal region of Rep68/40, LARGHSL, is similar to the atypical 14-3-3 binding motif, GHpSL, of the platelet glycoprotein (GP) Ib alpha (Bodnar et al., 1999). Our mutational and mass spectrometry analyses showed that at least the C-terminal ^{535}Ser of Rep68 is phosphorylated and that the 14-3-3 proteins specifically interact with Rep68 via phosphorylated ^{535}Ser.

In contrast to the direct interaction between Rep68 and 14-3-3 proteins, we did not detect an interaction between Rep78 and 14-3-3 proteins. This observation is consistent with the finding that 14-3-3 proteins associate with Rep68 through a unique C-terminal seven-amino-acid stretch shared by Rep68 and Rep40. No function or interacting proteins specific to this region have not been identified. Rep40 also harbors 14-3-3 binding site and interacts with 14-3-3 proteins (Fig. 2). Previous studies showed that the small Rep proteins, Rep52 and Rep40, increase the accumulation of single-strand AAV-2 genomes and are necessary for the efficient packaging of AAV-2 DNA (Chejanovsky and Carter, 1989; King et al., 2001). However, our mutant AAV-S535A, which also altered in Rep40's 14-3-3 binding site, did not show any change in the ratio of single-stranded and double-stranded replicated viral DNA. Thus, the role of 14-3-3 binding to Rep40 is currently unknown. On the other hand, the unique C-terminal region of Rep78, which is hydrophobic and contains a zinc finger motif, is known to be a binding target of protein kinase A and its homologues (Di Pasquale and Stacey, 1998). This region has been shown to be responsible for some functional differences between Rep78 and Rep68, such as cell cycle regulation (Saudan et al., 2000). Previous studies have suggested that Rep78 and Rep68 are differentially phosphorylated during the AAV life cycle (Collaco et al., 1997). We showed that the ^{535}Ser residue in the unique C-terminal region of Rep68 is phosphorylated, and that this phosphorylation is necessary for the binding of 14-3-3 proteins to Rep68. These observations may provide a clue to understand the unique role of Rep68, not shared with other Reps, in the AAV life cycle and cellular processes.

To evaluate the functional consequences of the interaction between Rep68 and 14-3-3 proteins, we examined the effects of 14-3-3 proteins on the DNA binding activity of Rep68. In gel-shift assays, DNA–protein complex that appeared in the Rep68/14-3-3–DNA reaction showed a similar mobility as that of a Rep68–DNA complex, but 14-3-3-bound Rep68 had a much reduced ability to initiate binding to both the ITR and *AAVS1* probes as compared with Rep68 alone. The apparent binding affinity of Rep68 for *AAVS1* (K_D of 5.5×10^{-9} M) is 2.5 times higher than that of a Rep68/14-3-3 ε complex (K_D of 1.4×10^{-8} M). Similarly, it has been shown that 14-3-3 proteins could influence the DNA binding affinity of the target proteins, either positively (p53, Waterman et al., 1998) or negatively (topoisomerase IIα, Kurz et al., 2000). The mechanism by which 14-3-3 proteins down-regulate the DNA binding of Rep68 is yet to be determined.

A mutant AAV encoding a Rep68 that lacks the 14-3-3 binding ability yielded more viral DNA than wild-type AAV in a DNA replication assay. Considering that ^{535}Ser-phosphorylated and 14-3-3-bound Rep68 protein had decreased DNA binding activity, loading and unloading of Rep68 on ITR and *AAVS1* is potentially regulated by its phosphorylation status and subsequent binding to 14-3-3 proteins during the AAV life cycle. This change in Rep68 DNA binding activity may also be involved in the regu-

lation of AAV DNA replication. Besides the AAV DNA replication, we also observed that the extent of the viral capsid proteins produced in the AAV-S535A mutant were higher that of the wild type (data not shown). Similarly, preliminary results examining the mutant viral particle production showed the higher rate than seen in the wild type (data not shown). These results strongly support the idea that 14-3-3 proteins are functionally involved in regulating the Rep68 protein's role during the viral life cycle. Another possible consequence of the association between Rep68 and 14-3-3 proteins is the alteration of the nuclear-cytoplasmic trafficking of Rep68, as suggested for other cellular proteins that bind to 14-3-3 proteins (Fu et al., 2000; van Hemert et al., 2001). When HeLa cells co-infected with AAV and adenovirus were fractionated into cytoplasmic and nuclear fractions, Rep78 was underrepresented in the cytoplasm but Rep68 was more prevalent (Im and Muzyczka, 1992). These results suggest a role for 14-3-3 proteins in modifying the subcellular localization of Rep68. Therefore, regulation of AAV DNA replication may also involve the control of Rep68-nuclear accumulation. Why is AAV DNA replication down-regulated by the binding of 14-3-3 to Rep68? Recently, it has been shown that Rep78 down-regulates adenovirus replication through PKA/PrKX binding (Di Pasquale and Chiorini, 2003). Rep68 may exert a regulatory effect over the helper virus replication through interaction with 14-3-3 proteins, and such interactions could be one of the controlling mechanisms for AAV to co-exist with the helper virus. We are currently examining whether the binding of 14-3-3 proteins to Rep68 is required for AAV replication fitness during adenovirus co-infection.

Materials and methods

Plasmids

The DNA fragments containing open reading frames of Rep68 and Rep78 from AAV-2 were obtained by PCR from pAV2 (Laughlin et al., 1983). The resultant PCR fragments were cloned into pBluescriptII SK+ to generate pBS-Rep68 and pBS-Rep78, respectively.

For expression in *E. coli*, the inserts were subcloned into the pET14b vector (Novagen). For expression in insect cells, pBS-Rep68 and pBS-Rep78 were subcloned into the baculovirus expression vector pFASTBAC HTc (Gibco BRL). The resultant construct would express either Rep68 or Rep78 tagged with six histidine (His) at the amino terminus. The N-terminal truncation mutant of Rep68 dl 1–102 Rep was generated by inserting a *Nco*I–*Not*I fragment (nucleotides 305–1611) of pBS-Rep68 into the pFASTBAC HTc. DNA fragments encoding dl 1–172 Rep and dl 1–224 Rep (Rep40) were amplified by PCR using pBS-Rep68 as the template, and inserted into the pFASTBAC HTb. The C-terminal truncated Rep68 mutant dl 371–536 Rep and dl 523–536 Rep mutant were constructed by inserting the *Spe*I–*Sal*I fragment (nucleotides 1–1108) and the *Spe*I–*Hin*dIII fragment (nucleotides 1–1562) of pBS-Rep68 into the pFASTBAC HTc.

All the point mutants of Rep68 were constructed by substituting the *Aat*II–*Not*I fragment (nucleotides 1548–1611) of pBS-Rep68 with double-stranded oligonucleotides. The respective Rep68 coding region was transferred using the *Spe*I–*Not*I site pFASTBAC HTc.

The cDNA fragments encoding 14-3-3 ε and 14-3-3 γ were obtained by PCR from a HeLa cell cDNA library. The PCR products were cloned into the pBSII SK+ plasmid (pBS-14-3-3 ε and pBS-14-3-3 γ).

DNA fragments containing 14-3-3 ε and γ were excised from pBS-14-3-3 ε and γ subcloned into pET14b modified to contain His-Flag sequences. The Flag-14-3-3 ε fusion gene was generated by PCR amplification of pBS-14-3-3 ε. HA-His-14-3-3 γ fusion was constructed by introducing the 14-3-3 γ encoding fragment into the HA-His-carrying vector pBSII SK+. The DNA fragments containing Flag-14-3-3 ε and HA-His-14-3-3 γ were then subcloned into the pFASTBAC1 (Gibco BRL), respectively.

The mutant AAV having the Rep68 substitution mutation (pAV1-S535A), whose nucleotide sequence of ^{535}Ser (tcg) was altered to Ala (gcg), was generated by PCR-based mutagenesis using pAV1 (Laughlin et al., 1983) as the template. KOD polymerase (TOYOBO) was used for all the PCR reaction according to manufacturer's instructions. All the sequences of the PCR-amplified DNA were verified by dideoxynucleotide sequencing analysis.

Expression and preparation of recombinant proteins

Expression of recombinant His-Rep68, His-Rep78, their derivatives, and Flag-14-3-3 ε, and HA-His-14-3-3 γ in insect cells (Sf9) was performed as described (Ishizu et al., 2001) using the pFASTBAC vectors and the BAC-TO-BAC system (Gibco BRL). The recombinant baculoviruses were used to infect SF9 cells at a MOI of 5–10 (Hoque et al., 1999). For co-infection, ratio of recombinant baculovirus to express His-Rep68 or His-Rep78 and Flag-14-3-3 ε or HA-His-14-3-3 γ were 1:1 and wild-type His-Rep68 or the S535A mutant, Flag-14-3-3 ε and HA-His-14-3-3 γ were 5:1:10. His-Rep68, His-Flag-14-3-3 ε, and His-Flag-14-3-3 γ were expressed from pET14b-based plasmids in the *E. coli* BL21 strain (or that carrying the pLysS plasmid for His-Rep68). All the recombinant proteins used in this study were tagged at their amino terminus.

The His-fusion proteins expressed in Sf9 and *E. coli* were purified by nickel chelate chromatography according to the manufacturer's instructions (Qiagen). The complex of His-Rep68 and Flag-14-3-3 ε used for gel-shift assay was prepared by two-step purification using anti-Flag M2 affinity gel (Sigma) with peptide elution and nickel chromatography from lysates of co-infected insect cells with baculovirus expressing His-Rep68 and Flag-14-3-3 ε.

Immobilization of Rep68 on the latex beads

The latex beads introduced with tosyl (*p*-toluene sulfonyl) groups for coupling polypeptides (SG-N-EOTs beads) were prepared as described previously (Hatakeyama et al., 1997). SG-N-EOTs (1 mg) beads were mixed with 20 μg of purified His-Rep68 in the immobilization buffer (10 mM HEPES-NaOH [pH7.9], 10% glycerol, 50 mM KCl, 1 mM EDTA). The coupling reaction was carried out at 4°C for 24 h in the dark. The latex beads were collected by centrifugation at 15,000 rpm for 5 min and washed three times with immobilization buffer. Remaining active esters were blocked by incubation in masking buffer (0.5 M Tris–HCl [pH8.0], 10% glycerol, 1 mM EDTA) at 4°C, for 12 h in the dark. The resulting beads were stored at 4°C. All manipulations were carried out on ice or at 4°C.

Affinity purification of Rep68-binding proteins

Nuclear extracts of the HeLa were prepared as described (Dignam et al., 1983). The Rep68-carrying latex beads (1 mg) were equilibrated 3 times with binding buffer (20 mM HEPES–NaOH [pH 7.9], 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.1% NP-40, 0.5 mM PMSF). The HeLa nuclear extract (about 450 μg of protein) in binding buffer was added to the latex beads and rotated at 4°C for 3 h. The binding reaction was terminated by centrifugation at 15,000 rpm for 5 min at 4°C to separate the latex beads from the supernatant. The latex beads were washed three times with binding buffer and then soaked twice in elution buffer (20 mM HEPES–NaOH [pH 7.9], 1 M KCl, 20% glycerol). Proteins were dissolved in SDS sample buffer, separated on a 10% SDS-PAGE, and visualized by silver staining.

Immunoprecipitation and immunoblotting

In vitro and in vivo binding assays of the Rep protein and the 14-3-3 protein were performed using anti-Flag M2 affinity gel according to the manufacturer's instructions with minor modifications. Briefly, 1–3 μg of purified His-Flag-tagged 14-3-3 ε or 14-3-3 γ was incubated with purified 1–3 μg of full-length or mutant Rep proteins for 2 h at 4°C for the in vitro binding assay. For in vivo binding assays, cell lysates were prepared from Sf9 cells co-infected with baculoviruses expressing the Rep and 14-3-3 proteins. Lysates were applied to the anti-Flag and incubated for 2 h at 4°C. After washing the resin with binding buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl [pH 7.9], 0.5% NP-40), bound proteins were eluted with SDS sample dye or excess Flag peptide and subjected to SDS-PAGE followed by immunoblotting using anti-Rep (Progen), anti-14-3-3 β (H-8), anti-14-3-3 ε (T–16), anti-14-3-3 γ (C–16) (Santa Cruz Biotechnology), anti-Flag, or anti-His (Sigma) antibodies.

For dephosphorylation, recombinant His-Rep68 protein purified from insect cells was treated with 0.02–2 units of calf intestinal alkaline phosphatase (Takara) for 30 min at 37°C before incubation with the 14-3-3 protein.

Mass spectrometry

About 20 μg of baculovirus-expressed and purified His-Rep68 was precipitated by acetone and digested with the Lys-C endopeptidase (Wako) for 4–12 h at 37°C in guanidine–HCl buffer or with Asp-N endopeptidase (Roche) for 5 h at 37°C in urea buffer. The resulting peptides were subsequently frozen and stored at −20°C. The digested peptides were purified on a reverse-phase column (Symmetry C18 5 μm, 0.32 × 150 mm, Waters, MA) using a capillary HPLC system (CapLC System, Waters). HPLC was carried out at a flow rate of 5 μl/min with a linear gradient of 5–45% CH_3CN in 0.1% formic acid over 80 min. The isolated peptides in the eluate were identified by mass measurements with a Q-TOF mass spectrometer (Micromass, UK) equipped with an electrospray interface (ESI) connected directly to the HPLC system.

Gel-shift assay and data evaluation

Plasmid pAV2 digested with *Bgl*II and *Hin*fI, releasing a 155-bp fragment containing the AAV ITR sequence, was blunt ended by the Klenow fragment and then subcloned into *Sma*I-digested pBS SK+ plasmid (pBS-155ITR). pBS-155ITR was then digested with *Bss*HII and the AAV ITR (nucleotides 18–103) was gel purified. The ITR probe used in this study lacks the first G residue of the first of the four GCTC repeats in the Rep binding sites (RBS). Approximately 20 ng of the fragment was labeled using Klenow fragment and [α-^{32}P]dCTP, followed by boiling and quick-chilling to allow formation of the hairpin ITR conformation. The secondary structure conformation of ITR was checked by nondenaturing polyacrylamide gel electrophoresis. *AAVS1*, fragment encompassing Rep recognition sequence (Weitzman et al., 1994), was generated by annealing of two oligonucleotides 5′-AATTCGGCGGTTGGGGCTCGGCG CTCGCTCGCTCGCTGGGCGGGCGG -3′, and 3′-G CCGCCAACCCCGAGCCGCGAGCGAGCGAGCGACC-CGCCCGCCCTAG-5′, and gel purified. Partial restriction enzyme sites, *Bam*HI and *Eco*RI, are underlined. Purified fragment was 5′end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Gel-shift assays were carried out as follows. The reaction mixtures containing 10 mM HEPES–KOH (pH 7.9), 5% glycerol, 50 mM KCl, 0.1 mM EDTA, 0.05% NP-40, 100 ng poly (dI–dC), 10 μg bovine serum albumin (BSA), the indicated amounts of purified His-Rep68 or His-Rep68/Flag-14-3-3 ε complex in a 10-μl final l final volume were incubated on ice 30 min. Approximately 0.1 ng of hairpin ITR or 0.02 ng of *AAVSI*-labeled DNA was then added and the reaction continued for a further 30 min

on ice. In competition experiments, indicated amounts of unlabeled *AAVSI* were added at this point. The reaction mixtures were separated by 4% or 6% nondenaturing polyacrylamide gel electrophoresis containing 0.5× TBE at 4°C for 1.5 h. The amounts of protein-bound and free reactants were quantified by measuring the radioactivity using STORM (Amersham Biosciences). The equilibrium dissociation constant (K_D) of Rep68 for its DNA binding sites was calculated by Lineweaver–Burk plot analysis as described previously (Suzuki et al., 1998).

Cell culture, transfection, and virus infection

HeLa cells were grown in monolayers at 37°C in 5% CO_2 in Eagle's minimal essential medium supplemented with antibiotics, glutamine, and 10% fetal calf serum. Semi-confluent HeLa cells were transfected with pAV1 or pAV1-S535A by effectene reagent (Qiagen), and then infected with adenovirus type 5 at a MOI of 10.

Southern blotting and quantitative PCR

Cells were harvested 72 h post-infection, and then viral DNA was extracted by a modified Hirt procedure (Hirt, 1967). The extracted DNAs were electrophoresed on a 1.2% agarose gel and transferred to a nylon membrane by capillary blotting in 0.4 M NaOH buffer. The filters were hybridized in hybridization buffer (5× SSC, 0.5% SDS, 100 μg of salmon sperm DNA) at 65°C for 12 h with an [α-^{32}P]dCTP-labeled probe generated by random priming using 1.8 kb *Pst*1 fragment of pBS-Rep78 used as the template. The filters were washed subsequently 2× SSC–0.1% SDS, 1× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS at 65°C for 10 min, and then analyzed by STORM and autoradiography.

The viral DNA was added to a quantitative PCR solution containing 1× SYBR Green master mix (Qiagen), 0.25 pmol/μl of forward and reverse primer as described (Di Pasquale and Chiorini, 2003). The amplified DNA was detected using iCycler (Bio-Rad). The thermoprogram was set to a 15-min denaturation at 95°C, and 50 times cycling reaction of 15 s at 94°C, 15 s 57°C, and 30 s 72°C. Known amount of purified AAV genome was used as the DNA standards.

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