AAV-2 Rep78 and HPV-16 E1 Interact *in Vitro*, Modulating Their ATPase Activity†

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ABSTRACT: Adeno-associated virus (AAV) is a nonpathogenic single-stranded human parvovirus which usually requires the presence of a "helper" virus for strong DNA replication. In addition to adeno- and herpes viruses, human papillomavirus (HPV) can serve as an AAV helper. We recently published that HPV type 16 (HPV-16) E1 protein contributes significantly as an individual helper gene for AAV-2 DNA replication and transcription. As Rep78 and E1 are the corresponding DNA helicase/replication proteins of AAV and HPV, respectively, and Rep78 and E1 have a degree of homology, we assayed whether these two proteins interact physically. The full length proteins were purified from bacteria as GST-E1 and MBP-Rep78 and used in five assays to observe Rep78−E1 interactions. All five assays (pull-down, coimmunoprecipitation, enzyme-linked immunosorbent assay (ELISA), chemical cross-linking, and ATPase activity) provided evidence consistent with Rep78−E1 interaction. Most intriguing, an overall decrease in ATPase activity was observed when both proteins were present together. These data strongly suggest that E1 and Rep78 interact and that this interaction modulates at least some of their individual biochemical functions. This study adds to our understanding of AAV-HPV interaction biology, E1's modulation of Rep78 biochemistry, Rep78's modulation of E1 biochemistry and provides initial clues which may lead to the underlying mechanism of HPV E1 helper function for AAV DNA replication.

Adeno-associated virus (AAV)[11] is a nonpathogenic, helper dependent, single stranded DNA human parvovirus. The genome of AAV contains two large open reading frames (ORF) including the *rep* and the *lip-cap* genes and the palindromic inverted terminal repeat elements (ITR) (*1*). While AAV can replicate autonomously under specific situations (*2*−*5*), it usually requires co-infection with a helper virus for productive infection. Adenovirus, herpesvirus, and papillomavirus can serve as helpers for AAV (*6*, *7*). In absence of any helper virus, AAV can establish latency by integrating site specifically on the human chromosome 19 (termed AAVS1) (*8*). Epidemiological studies (*9*−*12*) suggest that AAV plays a protective role against the development of genital cancers. Genetic analysis of AAV genome suggests that the majority of the antioncogenic property of AAV can be attributable to the full-length product of the AAV *rep* gene, the Rep78 protein (*13*−*19*). Rep78, the major replication protein of AAV, is a multifunctional protein which has an ATP-dependent helicase and endonuclease activity (*20*, *21*). It has the ability to both *trans*activate and *trans*repress AAV's own promoters (*18*, *22*−*26*). Several heterologous promoters, including viral and proto-oncogene promoters, are also known to be inhibited by Rep78 (*16*, *18*, *27*, *28*). Being a transcription factor, Rep78 has been found to bind several proteins including Sp1 (*29*), TBP (*30*, *31*), and PCNA (*32*) and also to itself (*17*, *33*). Recently, AAV has become noted for its extensive use as a gene therapy vector (*34*−*36*).

Human papillomaviruses (HPVs) are double stranded DNA viruses which belong to the papovaviridae family (*37*). In contrast to AAV, HPVs are strongly implicated in cervical cancer (*38*). While virally encoded E6 and E7 are responsible for oncogenic transformation (*39*, *40*), E1 and E2 proteins are essentially required for HPV replication. E2 is a multifunctional transcription and replication factor (*41*, *42*). E1 is a phosphoprotein which is the ATP-dependent viral DNA helicase (*43*−*46*), binds to the HPV origin of replication (*ori*) within the viral URR (upstream regulatory region) (*47*), and initiates HPV replication by forming a double hexamer in the *ori* (*48*).

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[1] Abbreviations: AAV, adeno-associated virus; HPV, human papillomavirus; GST, glutathione S-transferase; MBP, maltose binding protein; ELISA, enzyme-linked immunosorbent assay; ITR, inverted terminal repeat; ATP, adenosine triphosphate; TBP, TATA binding protein; PCNA, proliferating cell nuclear antigen; URR, upstream regulatory region; m.o.i, multiplicity of infection; SV40, simian virus 40; ORF, open reading frame; IPTG, isopropyl β-D-galactopyranoside; HSV, herpes simplex virus; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; TBST, Tris-buffered saline with Triton-X 100; TMB, 3,3′,5,5′-tetramethylbenzidine; PVDF, polyvinylidene difluoride; BME, β-mercaptoethanol; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TLC, thin layer chromatography; PEI, polyethyleneimine; BS^3, bis(sulfosuccinimidyl) suberate;

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AAV, like HPV, is also a genital virus (*49*) and the existence of bidirectional interaction between HPV and AAV are well established (*12*, *50*−*57*). However, while AAV and HPV are both part of the genital flora, AAV may be negatively associated with cervical cancer, while HPV is the major risk factor for it. Moreover, HPV provides helper function for AAV productive replication *in vitro*, and, in turn, AAV inhibits HPV-induced oncogenic transformation. In cultured raft skin equivalents, at low multiplicities of infection (m.o.i), AAV actually boosts HPV replication, but a high m.o.i of AAV inhibits HPV (*56*). In any case, the individual HPV helper genes may be useful for rAAV production as the adeno- and herpes virus genes have proven to be. Also, this bidirectional interaction of AAV-HPV has important consequences on cancer prevention and gene-therapy.

Previous studies from our laboratory has demonstrated that HPV E1 provides a helper effect for AAV replication (*52*) though the specific mechanism of action is unknown. One possibility is that HPV E1 might directly interact with AAV Rep78, both being the respective replication proteins of the two viruses. As mentioned earlier, Rep78 is well demonstrated to bind a variety of viral (HSV ICP8, Ad E1a) (*58*, *59*) and cellular proteins (like RPA, TBP, Sp1) (*29*, *30*, *60*), including HPV E2 (*60*, *61*) and E7 (*50*). The sequence homology among different domains of Rep78 and E1 proteins has been described in many studies (*62*, *63*, *81*). Most intriguing is that Rep78 binds to SV40 Large T antigen, again, the equivalent replication protein of SV40 (*64*), which also shares a number of biochemical properties with both AAV Rep78 and HPV E1.

The objective of the present study was to investigate if AAV Rep78 and HPV E1 physically interact. Here we demonstrate, for the first time, a direct interaction between Rep78 and E1 *in vitro* using multiple assays (pull-down, ELISA, coimmunoprecipitation, chemical cross-linking, and ATPase assay). We also show that the interaction between Rep78 and E1 modulates ATPase activity of these proteins. The implication of this interaction is still not clear but it could be predicted that this interaction by modulating their own biochemical activity, at least by part, will help to explain the bidirectional interaction of HPV and AAV, aid in an understanding of AAV's inhibition of HPV, and help to explain the helper effect of E1 for AAV.

MATERIALS AND METHODS

Antibodies. Anti-GST antibody (Cat no. RPN 1236V) and Anti-MBP antibody (cat no. E8038S, E8032S) were purchased from Amarsham Pharmacia and New England Biolabs, respectively.

Protein Purification. Purification of MBP-Rep78 was described previously (*65*). Briefly, the entire Rep78 ORF was cloned in-frame with the maltose binding protein (MBP) ORF in the pMALc2 (New England Biolabs protein fusion and purification system). Purification of MBP-Rep78 protein was performed using protein purification and expression system (New England Biolabs) following kit directions. Fractions were collected and concentrated using centricon filters (Amicon). The expression vector for full length GST-E1 was kindly provided by Peter Howley. GST fusion protein was purified as described elsewhere (*50*) with some modifications. The plasmid was transformed into BL21(DE3), and the bacteria were grown to an optical density of 0.6 before being induced with IPTG (isopropyl β-D-galactopyranoside) at 0.5 mM for another 3 h at 37 °C. GST-E1 fusion protein was purified with the GST purification module (Amersham Pharmacia Biotech). Briefly, the bacterial culture was centrifuged and resuspended in PBS with protease inhibitors. The bacteria were lysed by sonication (setting 5, three 30 s interval on ice). The fusion protein was applied to a glutathione-Sepharose column (Amarsham Pharmacia), and after washing, GST-E1 was eluted in reduced glutathione (supplied with the kit). Quality of the both proteins was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Commassie blue staining following the standard protocol.

Protein Affinity Pull-Down Assay. GST and GST-E1 proteins were bacterially expressed and bound to glutathione-Sepharose. The beads were washed and resuspended as a 50% (vol/vol) suspension in suspension buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 10%glycerol, 1 mM dithiothreitol, 0.5 mM phynylmethylsulfonyl fluoride). For the precipitation assay, the beads were blocked overnight with 2.0% (wt/vol) bovine serum albumin (BSA) in phosphate-buffered saline. Following the removal of BSA, MBP-Rep78 protein was added to the 50 μL of Sepharose bead suspension. The final volume was adjusted to 0.5 μL with suspension buffer supplemented with 0.05% [vol/vol] NP-40, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The tubes were rocked at 4 °C for 3 h, and the beads were precipitated by centrifugation and washed five times with the same buffer. Following the last wash, the supernatant was completely removed, and the beads were resuspended in 20 μL elution buffer. A 100 μL amount of last wash solution was saved for analysis.

For analysis, samples were separated on 10% SDS-PAGE gels and transferred electrophoretically to nitrocellulose membranes. These membranes were blocked overnight with 5% (wt/vol) dry milk in TBST. The membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-MBP antibody. Bound proteins were visualized by chemiluminescence detection reagent (Pierce). The assay was repeated at least three times. For every experiment it was ensured that the amount of GST bound to the matrix was equal or higher than the amount of GST-E1.

In the reciprocal pull-down experiment, MBP or MBP-Rep78 proteins were bacterially expressed, bound to amylose resin (New England Biolabs) and used to precipitate GST-E1. Bound protein was detected by anti-GST antibody following the same method as above.

Immunoprecipitation of GST-E1 with MBP-Rep78. Protein A/G plus-agarose (Santa Cruz Biotechnology, Inc.) was used for the precipitation analysis. Equimolar amount of MBP-Rep78 and GST/GST-E1 was incubated at 4 °C for 3 h in suspension buffer (20 mM Tris-HCL [pH 7.4], 100 mM NaCl, 10%glycerol, 1 mM DTT, 0.5 mM PMSF, 1 mM $CaCl_2$, 1 mM $MgCl_2$). The solution was incubated overnight with 20 μL of protein A/G-agarose beads that had been conjugated with anti-MBP antibody. The beads were washed twice with the same buffer and twice with PBS, boiled in 20 μL of SDS-PAGE sample buffer, and analyzed by SDS-PAGE on 10% polyacrylamide gels (Gene Mate Express gels, ISC Bio Express). The separated components were

transblotted onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked overnight with 5% (wt/vol) dry milk in TBST, incubated with HRP-conjugated anti-GST antibody, and visualized by chemiluminescence detection reagent (Pierce). The assay was repeated at least thrice.

ELISA-Based Protein Interaction Assay. ELISA was carried out in 96-well vinyl plates at room temperature following the method described by Han et al. (1999) (*66*). Wells were coated for 1 h with purified MBP-Rep78/GST-E1 in 50 μL of TBS (25 mM Tris-HCL, pH 7.4; 150 mM NaCl). The wells were then washed with TBST (TBS with 0.1% [vol/vol] NP-40 or Triton X-100) and blocked with 190 μL of dry milk (5 wt %/vol) and calf serum (2% vol/vol) in TBST for 1 h. After washing with TBST, various amounts (as described in the figure legend) of purified GST/GST-E1 and MBP/MBP-Rep78 in 50 μL of TBST were added to the MBP-Rep78- and GST-E1-coated plates, respectively, and incubated for 1 h. The plates were then washed in TBST and incubated for 1 h with horseradish peroxidase-conjugated anti-GST/anti-MBP antibody diluted in TBST with 0.5% dry milk and 1% calf serum. After being washed five times with TBST and four times with TBS, the wells were incubated with 50 μL of visualization buffer (110 mM sodium acetate [pH 5.5] containing 0.02 mg of TMB/mL and 0.0075% hydrogen peroxide). After 10 min the reactions were stopped by the addition of an equal volume of 2 M sulfuric acid. The assays were then quantified spectrophotometrically by absorbance at 450 nm. The assay was performed in duplicate and repeated at least thrice.

In Vitro Protein Cross-Linking Assay. In a final volume of 20 μL reaction buffer (20 mm HEPES, pH 7.5; 4 mM $MgCl_2$, and 4 mM ATP) MBP-Rep78 and GST-E1 were cross-linked with 4 mM BS^3 [bis(sulfo succinimidyl)-suberate}, Pierce] for 30 min or 5 min at room temperature. Amount of proteins and reaction conditions were as indicated in the picture. The reaction was terminated by 1 M glycine for 15 min, run in a 3−8% SDS-PAGE gel, transferred on polyvinylidene difluoride (PVDF) membrane, and hybridized with HRP-conjugated anti-GST antibody (1:10 000 dilution) or anti-MBP antibody (1:10 000 dilution). In some of the experiments, the blots were stripped off in 100 mM β-ME, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7 at 56 °C for 1 h, blocked overnight, and rehybridized with anti-MBP or anti-GST antibody.

ATPase Assays. ATPase assays were carried out according to the protocol described by Wonderling et al. (1995) (*67*) with some modifications. In a final volume of 10 μL containing 25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$,1 mM DTT, 100 μg BSA/mL, 0.066 μM of [α-^{32}P]-ATP (3000 Ci/mmol) and different concentrations of the proteins (indicated in the figure). The reaction mixtures were incubated at 37 °C and the products were analyzed by thin layer chromatography (TLC). At 3 min interval, one microliter of the reaction mixture was taken out and spotted onto plastic-backed polyethyleneimine (PEI)-cellulose sheets (EM Sciences, Gibbstown, N.J). The reaction was terminated by the addition of EDTA to a final concentration of 20 mM. The sheets were developed by ascending chromatography in 1.0 M formic acid and 0.5M LiCl, dried and scanned by Molecular Imager (Bio Rad). Quantification was done by the Quantity One software.

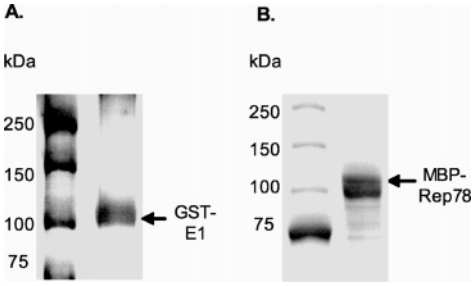

FIGURE 1: Fusion proteins purified from *E. coli*. GST-E1 and MBP-Rep78 were expressed and purified as described in Materials and Methods, separated by SDS-PAGE, and stained with Coomassie blue following standard protocol. The positions of the molecular mass markers are indicated on the left of the each gel.

RESULTS

In Vitro Interaction of Rep78 and E1 by Pull-Down, Immunoprecipitation, and ELISA. E1, the replication protein of HPV, has been shown to provide the helper function for AAV replication. We have previously hypothesized that E1 might interact with Rep78, the replication protein of AAV, because of their similarities in function. To identify this interaction, affinity pull-down analysis was done first. Full length HPV E1 and AAV Rep78 were expressed in bacteria as fusion proteins in frame with GST (Figure 1A) and MBP (Figure 1B), respectively, purified, and tested for their ability to interact *in vitro* by pull-down assay. For ease of discussion, hereafter, we will refer to GST-E1 and MBP-Rep78 as E1 and Rep78, respectively. Bacterially expressed GST or E1 was purified on glutathione sepharose beads. These beads were then incubated with purified Rep78 protein at 4 °C for 3 h. After five sequential washings, the bound protein was eluted in elution solution. The load, wash, and eluted solution were analyzed for the presence of Rep78 by Western blotting with anti-MBP antibody. As observed in a representative experiment (Figure 2A), Rep78 was strongly detected in eluate from the E1 column, while only trace amounts of Rep78 could be detected (as shown in Figure 2A) in eluate from GST-alone column. These data suggest an interaction of Rep78 with E1.

The pull-down assay was also performed in reverse orientation. Purified E1 was applied to MBP or Rep78-bound amylose resin. After extensive washing, the bound proteins were eluted. The load, wash, and eluate were analyzed for the presence of E1 by Western blotting and probing with anti-GST antibody. As shown in a representative experiment (Figure 2B) E1 could be detected in the eluate from the Rep78 column. In some of the experiments E1 could be detected also in MBP columns, but the amount of E1 eluted from MBP columns was always only a small fraction of the amount eluted from Rep78 columns. These results strongly suggested that Rep78 binds to immobilized E1 on a matrix and vice versa.

Rep78 was also tested for its capacity to interact with E1 by immunoprecipitation from solution. MBP-Rep78 and GST-E1 were incubated in suspension buffer, and Rep78 protein was precipitated with anti-MBP-conjugated protein A/G-agarose. After elution in the sample buffer, the precipitated proteins were separated by SDS-PAGE and detected by immunoblotting with anti-GST antibody. As shown in a representative experiment (Figure 3, one of four), E1 could

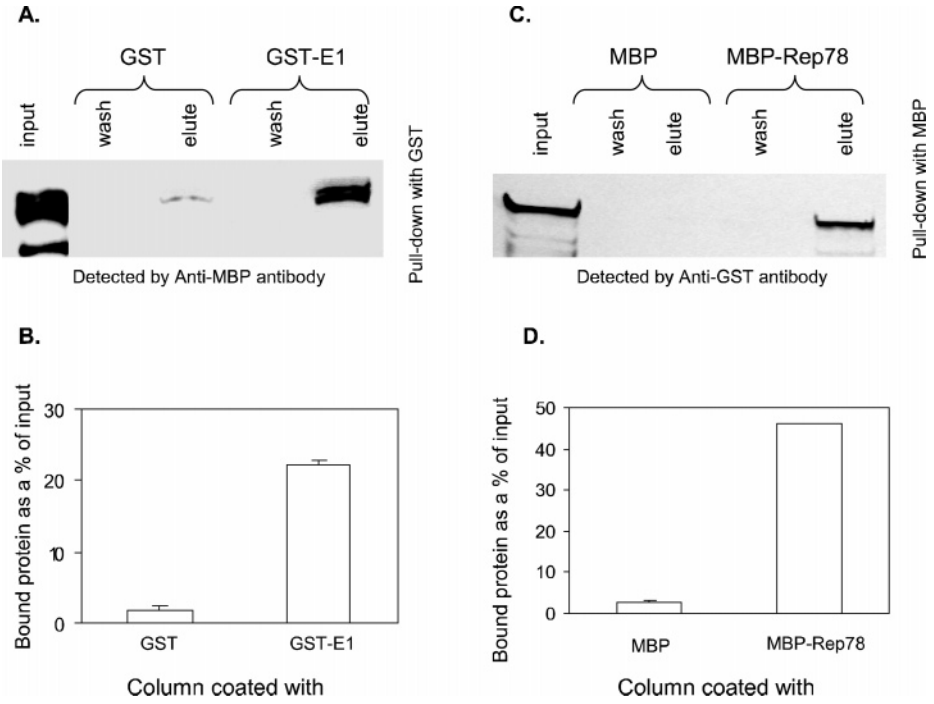

FIGURE 2: Rep78 binds E1 by pull-down assay. (A) The pull-down assays were performed as described in Materials and Methods by incubation of bacterially expressed GST or GST E1 protein-bound glutathione Sepharose beads with purified MBP-Rep78 protein for 3 h at 4 °C. The beads were precipitated by centrifugation and washed, and the proteins were eluted. The various samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, and detected by polyclonal rabbit anti-MBP antibody. The lane marked 'Input' consists of 10% of MBP-Rep78 used in each precipitation that was directly subjected to SDS-PAGE without precipitation. The lane marked 'GST' contains protein precipitated using GST-bound glutathione Sepharose beads. The lane marked 'GST-E1' contains protein precipitated using GST-E1-coated glutathione Sepharose beads. (B) Quantitative measurement of the bound protein as a percent of the input protein. (C) In a similar assay, bacterially expressed MBP or MBP-Rep78 proteins were purified in amylose resin. These resins were incubated with purified GST-E1 protein. The resins were precipitated by centrifugation and washed, and the proteins were eluted in elution buffer. The various samples were separated by SDS-PAGE, transferred to nitrocellulose membrane, and detected by anti-GST antibody. The lane marked 'Input' consists of 10% of GST-E1 used in each precipitation that was directly subjected to SDS-PAGE without precipitation. The lane marked 'MBP' contains protein precipitated using MBP-bound amylose resin. The lane marked 'MBP-Rep78' contains protein precipitated using MBP-Rep78 bound amylose resin. (D) Quantitative measurement of the bound protein as a percentage of the input protein. All experiments were repeated at least three times. The results depicted here were taken from a representative experiment.

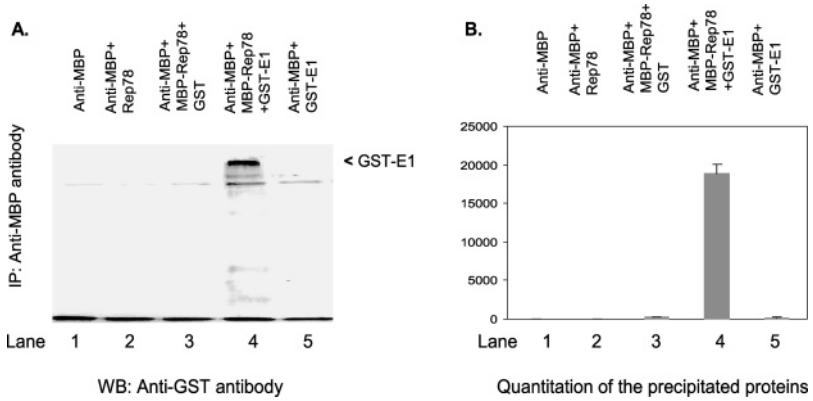

FIGURE 3: Coimmunoprecipitation of E1 with Rep78 by anti-MBP antibody. Rep78 and E1 proteins were incubated together and coprecipitated from solution by anti-MBP antibody bound proteinA/G-agarose. Analysis of the precipitated materials was done by electrophoresis and Western blotting with anti-GST antibody. (A) Western blot: Lane 1, anti-MBP; lane 2, MBP-Rep78 + anti-MBP; lane 3, MBP-Rep78 + GST + anti-MBP; lane 4, MBP-Rep78 + GST-E1 + anti-MBP; lane 5, GST-E1 + anti-MBP. Precipitation experiments were repeated at least four times. The results depicted here were taken from a representative experiment. (B) Quantitative estimation of the precipitated material.

be coprecipitated with Rep78 but not alone by anti-MBP antibody, again strongly suggesting Rep78−E1 interaction.

ELISA-based protein interaction assay was used to further confirm the binding of Rep78 and E1. Purified Rep78 was immobilized in the ELISA wells which were then blocked. Increasing amounts of GST and E1 proteins were applied to the wells. Unbound proteins were then removed by washing, and that GST or GST E1 remained bound to the Rep78 was detected by anti-GST antibody. Binding of E1 to Rep78 increased with the amount of E1 added (Figure 4A) whereas binding of GST to Rep78 remained at background levels. The reciprocal ELISA, adding increasing amount of MBP/Rep78 on immobilized E1, also demonstrated an interaction (Figure 4B) between Rep78 and E1. The interaction of the proteins was detected by anti-MBP antibody. The interaction of Rep78 with E1 was much higher than the interaction of

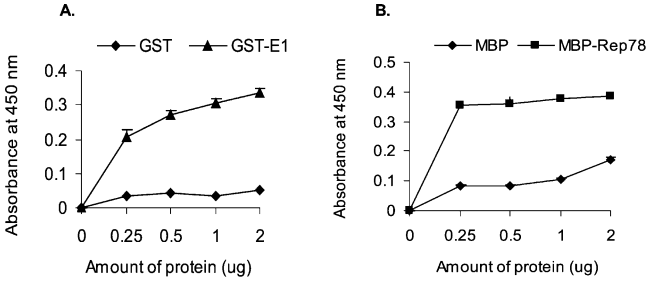

FIGURE 4: Rep78 binds E1 by ELISA-based protein interaction assay. (A) GST-E1 binding to immobilized MBP-Rep78. ELISA was performed in which purified MBP-Rep78 (1 μg) protein was immobilized in 96-well plates and then challenged with increasing amounts of bacterially produced GST and GST-E1. The interaction was detected by the retention of GST/GST-E1 as detected with goat polyclonal antibody against GST conjugated with horseradish peroxidase. The wells were incubated with a chromogenic substrate, and absorbance was measured at 450 nm as described in Materials and Methods. (B) MBP-Rep78 binding to immobilized GST-E1. A 1 μg amount of GST E1 was bound to ELISA wells and challenged with increasing amounts of purified MBP or MBP-Rep78 proteins. After washing, retained MBP/MBP Rep78 was detected with anti-MBP antibody.

MBP with E1 and was consistent with the pull-down and immunoprecipitation assay results.

Rep78 and E1 Can Be Cross-Linked in Vitro. Although the pull-down and ELISA assays strongly suggested Rep78−E1 interaction *in vitro*, it was further investigated whether a chemical cross-linker could cause intermolecular cross-linking between Rep78 and E1, as both E1 and Rep78 are known to form homooligomers. For cross-linking assay, the homobifunctional lysine cross-linking agent BS^3 was chosen because of its high selectivity, moderate spacer length, aqueous solubility, and wide precedence in the literature. It reacts with amino groups in the target protein and is suitable for intermolecular cross-linking. The fusion proteins were used without cleaving the GST or MBP domain because of the easy detection of the cross-linked products by commercially available anti-GST or anti-MBP antibody. Figures 5−8 show representative Western blots of Rep78 and E1 cross-linked with BS^3 under different conditions and blotted with anti-MBP or anti-GST antibody. Incubation of Rep78 with BS^3 generated several high molecular weight cross-linked products (Figure 5A) at different conditions. It is important to note that a difference in cross-linking band pattern was observed with the addition of E1 (lanes 1 & 4) in the presence of ATP-Mg^{2+}. This condition was used in further assays. Incubation of a fixed amount of Rep78 with BS^3 and increasing amount of GST−E1 or a fixed amount of E1 with increasing amount of Rep78 reproducibly led to the appearance of additional immunoreactive bands (Figure 5B), indicating that cross-linking occurred between Rep78 and E1. But the size of these large complexes could not be accurately estimated. Most of the proteins ran as high molecular mass complexes that barely entered the gel. These high molecular mass forms could be detected by both Western blotting and Commassie blue staining (data not shown).

The extent of cross-linking depends on both the time of incubation and the concentration of BS^3. However, no significant band changes were observed at lower BS^3 concentrations (data not shown). But at reduced time, cross-linked products of lower molecular mass could be detected more clearly (Figure 6A). As Western blotting was used to detect the cross-linking products, it was difficult to determine the precise molecular mass of the large Rep78−E1 complexes. A new complex of higher order oligomers appeared with the addition of E1. Furthermore, the intensity of the band was increased with the increasing amounts of E1. So it could be suggested and supported that E1 and Rep78 might be forming heterooligomeric complexes. The band intensity of the lower order oligomers was also increased in the presence of E1 but remained constant with increasing amount of E1. Probably, presence of E1 was interacting with Rep78, promoting it to form more lower-order associations without being involved in it. Appearance of new additional bands was not observed when increasing amounts of GST were cross-linked with a fixed amount of Rep78 (Figure 6B). The blot of Rep78−E1 cross-linking was stripped off and reprobed with anti-GST antibody (Figure 6C) which confirmed the presence of E1 in lower molecular mass cross-linked product in a dose-dependent manner. The two blots probed with anti-MBP and anti-GST antibody were superimposed and the distance of the complex from the Rep-only band was measured. Presence of superimposable bands (Figure 6D) in both blots confirmed the presence of both Rep78 and E1 in the indicated complexes, again directly and strongly suggesting heterooligomeric complexing between Rep78 and E1.

To ensure that intermolecular cross-linking was due to E1-Rep78 interaction and not caused by the MBP motiety-to-GST-E1 or GST moiety-to-MBP-Rep78 cross-linking, multiple control assays were undertaken. The cross-linking of GST and MBP was done in the presence of Rep78 (Figure 7A) and E1 (Figure 7B), respectively. Though a self-association of GST and MBP was observed at this concentration of BS^3, the vast majority remained monomeric, indicating that GST and MBP did not self-associate significantly. More importantly, any additional band or change in cross-linking pattern was not observed in the presence of Rep78 (Figure 7A) or E1 (Figure 7B) which confirmed that GST or MBP moiety did not participate in the oligomerization of Rep78 and E1.

In a similar but in reverse orientation cross-linking assay, E1 and Rep78 was cross-linked by BS^3 in different conditions and immunoblotted with anti-GST antibody (Figure 8A). Here also, a difference in the cross-linking pattern was clearly observed in the presence of Rep78 and ATP-Mg^{2+}. But the specific high molecular complexes could not be resolved. Figure 8B demonstrated the cross-linking of a fixed amount of E1 with BS^3 and increasing amounts of Rep78 for 5 min and immunoblotted with anti-GST antibody. It was observed that amount of low molecular mass complexes increased in parallel with increasing amount of Rep78. The blot was stripped off and reblotted with anti-MBP antibody (Figure 8C) which ensured the presence of Rep78 in lower molecular mass complexes. As a whole, the results presented in Figures 5−8 demonstrated that Rep78 and E1 could be cross-linked *in vitro* by BS^3 and that they are forming a heteromultimeric complex.

Modulation of Overall ATPase Activity of Rep78 and E1. As Rep78 and E1 interact with each other, their functional activity might be affected. To address this question, the ATPase activity, an essential function of both Rep78 and E1 for AAV and HPV replication, respectively, was com-

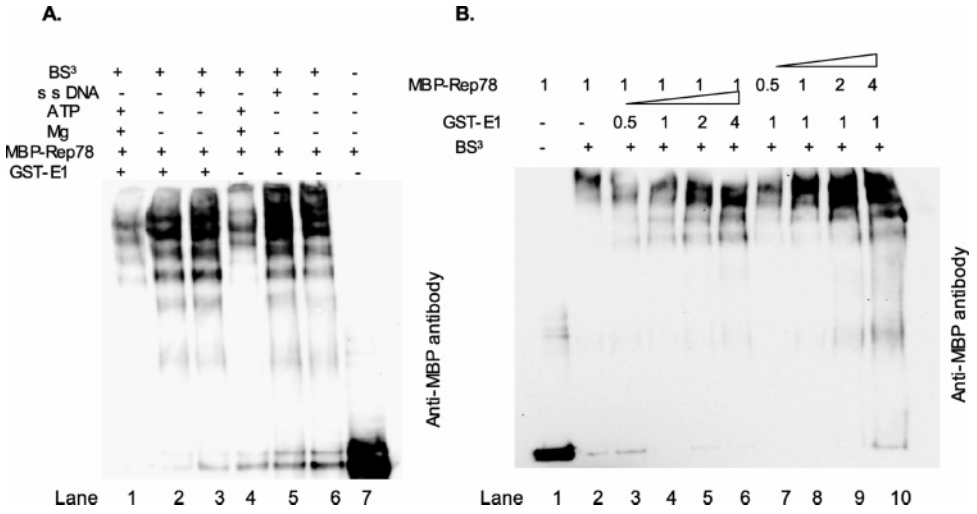

FIGURE 5: Multiple Rep78−E1 complexes are captured by chemical cross-linking assay. Cross-linking was performed for 30 min with 4 mM BS^3 at room-temperature. The reaction was terminated by 1 M glycine for 15 min, run in a 3−8% SDS-PAGE gel, transferred on PVDF membrane, and hybridized with HRP-conjugated anti-MBP (1:10 000) antibody. The experiments were repeated at least three times. The result depicted here was taken from a representative experiment. (A) A 1 μg amount of MBP-Rep78 was incubated with/without 1 μg of GST-E1 and BS^3 at room temperature for 30 min. Mg^{2+}, ATP, and ssDNA were added to the reaction buffer as indicated. A difference in the cross-linking pattern of Rep78 (in the presence of ATP and Mg) was observed with addition of E1. (B) A fixed amount (1 μg) MBP-Rep78 was cross-linked with an increasing amount (as indicated) of GST-E1 or a fixed amount of GST-E1 (1 μg) with an increasing amount of MBP-Rep78 (as indicated) with BS^3 for 30 min, separated on a SDS-PAGE gel, transferred to PVDF membrane, and hybridized with anti-MBP antibody. A set of low molecular mass oligomers could be seen in the presence of E1.

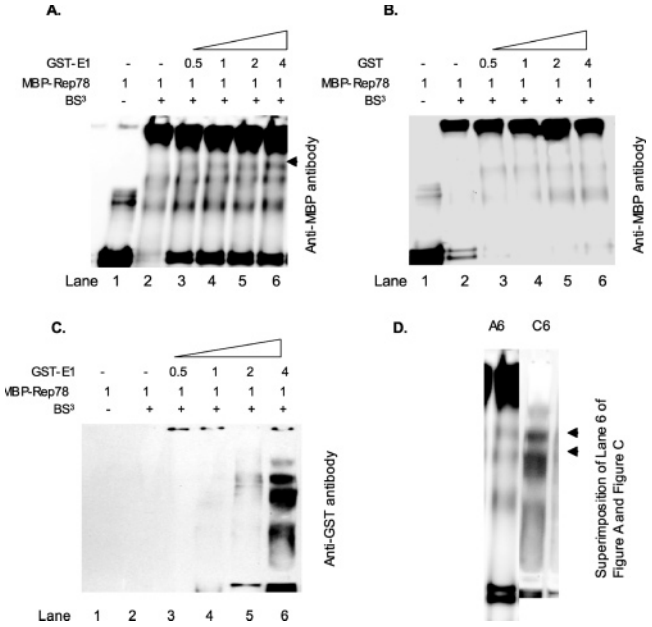

FIGURE 6: Simultaneous presence of Rep78 and E1 in specific complexes. The cross-linking reaction was repeated with 1 μg of MBP-Rep78 and BS^3 in the presence of an increasing amount of GST-E1 (A) or GST (B) for 5 min. The products were resolved on a SDS-PAGE gel, transferred to PVDF membrane, and immunoblotted with anti-MBP antibody. A new set of bands could be seen in the presence of E1. The same blot used in Figure A was stripped of and blotted with anti-GST antibody (C). It confirmed the presence of E1 in the complexes. Superimposition of lane 6 of Figure A and lane 6 of Figure C results in contiguous bands (D) which ensured the cross-linking of MBP-Rep78 and GST-E1.

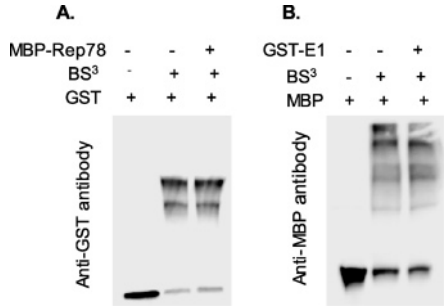

FIGURE 7: GST and MBP moieties do not participate in the cross-linking of GST-E1 and MBP-Rep78. (A) A 1 μg amount of GST was cross-linked with/without 1 μg of MBP-Rep78 and probed with anti-GST antibody as described in Materials and Methods. No new band appeared upon addition of MBP-Rep78. (B) A 1 μg amount of MBP was cross-linked with/without 1 μg of GST-E1 and probed with anti-MBP antibody as described in Materials and Methods. No new band appeared upon addition of GST-E1.

pared using an equimolar amount of Rep78 and E1. ATPase assay was performed by measuring the release of inorganic phosphate as described in Materials and Methods. Figure 9A represents representative TLC plates of ATP hydrolysis with time by the individual proteins compared to that of the protein together. An increase in ATPase activity with time is clearly observed for each of the protein or the protein together. Figure 9B is the endpoint estimation of the percent ATP hydrolyzed after 40 min which confirmed that the GST or MBP moiety was not contributing to the ATP hydrolysis. Figure 9C graphically compares the ATPase activities of the proteins at different time interval. "Estimated" and "experimental" ATPase activity refers to the summation of their individual activity and the actual ATPase activity when they were present together, respectively. The 'experimental' ATPase activity was found to be less than the "estimated" ATPase activity.

Under the conditions used, the ATPase activity of E1 was doubled ($p < 0.05$, all time points except at 6 min) with doubling of the E1 protein. In contrast, the ATPase activity of Rep78 was significantly less than double when the amount of Rep78 was doubled ($p < 0.05$, all time points). Most importantly, when equimolar amounts of E1 (1X) and Rep78 (1X) were used the overall ATPase activity (experimental) was significantly less than the equivalent (2X, double)

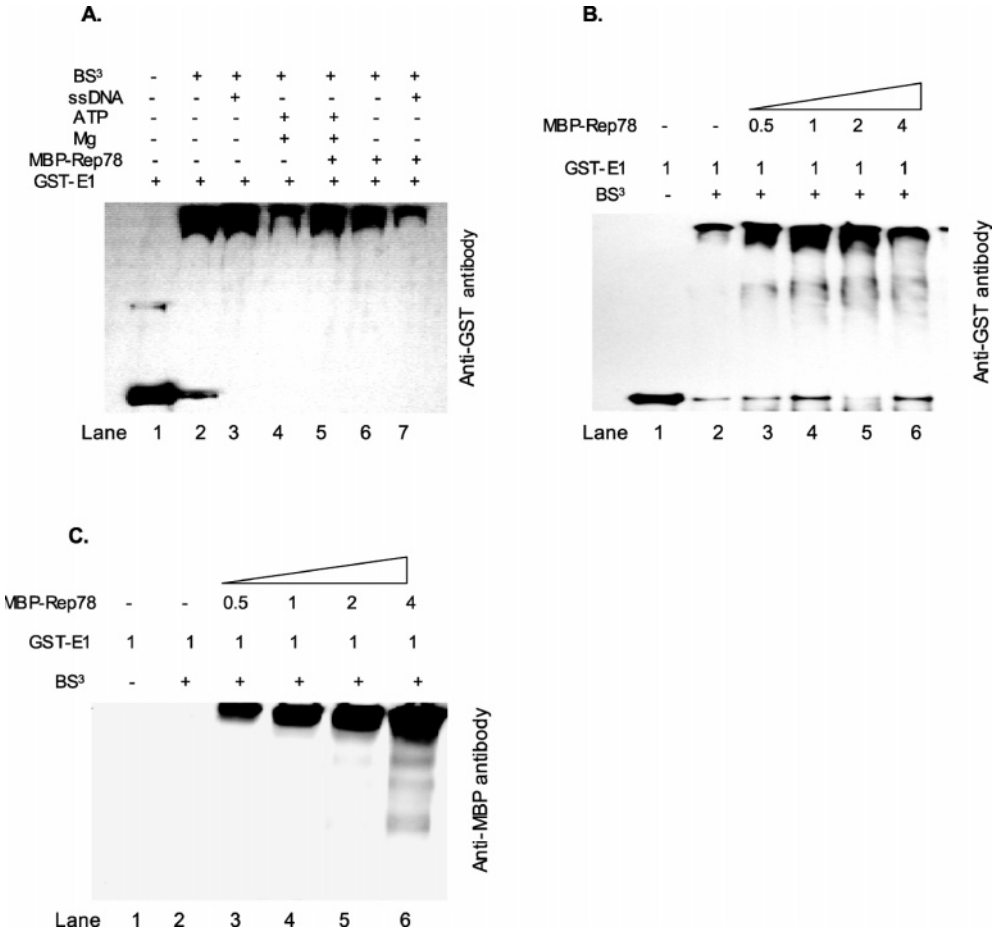

FIGURE 8: Reciprocal cross-linking analysis of Rep78−E1 binding. (A) A 1 μg amount of GST-E1 was cross-linked with BS^3 in the presence of MBP-Rep78 as indicated (μg) for 30 min. The molecular mass of the cross-linked products were indistinguishable. (B) Cross-linking was repeated for 5 min, and the reaction mixtures were gel separated and detected with anti-GST antibody. Complexes of lower molecular mass appeared with the addition of increasing MBP-Rep78. (C) The same blot was stripped off and reblotted with anti-MBP antibody which confirmed the presence of MBP-Rep78 in lower molecular mass complexes.

amount of Rep78 protein ($p < 0.05$, all time points). Finally, the Rep78−E1 combination was similar to double E1 protein ($p > 0.05$, all time points). The "experimental" ATPase activity of these two proteins was also less than their "estimated" ATPase activity. This last data, as well as Rep78−E1 activity being less than 2XRep78, further support the *in vitro* interaction of Rep78 and E1. No modulation of ATPase activity could be expected unless the two proteins interact. If there was no interaction, their combined ATPase activity (experimental) should be equal/comparable to the addition of their individual ATPase activities. These data also strongly suggest that the heterooligomer is less active than Rep78 homooligomers. These data also uncover a difference between Rep78 and E1 in regards to their ATPase activity, with Rep78 displaying self-inhibition upon doubling of the protein in contrast to E1, which did not exhibit this. The rationale behind using the two different concentrations of each protein was to check whether, under our experimental conditions, doubling the amount of protein doubled up the ATPase activity of the proteins so that we could compare the ATPase activity of these two proteins in the linear range. Also, when we compared the ATPase activities of the two proteins together, the activities of the equal number of molecules was compared.

DISCUSSION

In this study we investigated the ability of Rep78, the major replication and regulatory protein of AAV, to associate with another viral replication initiator protein and replicative helicase, HPV E1. Previous study (*52*) from our group identified that E1 has a significant helper effect on AAV and that it in turn increases the Rep78 expression at the mRNA level. This study investigates the possible physical interaction of Rep78 and E1 *in vitro*. Here we have demonstrated by five different assays that Rep78 and E1 directly interact and form heterooligomeric complexes *in vitro*. This interaction is accompanied by a reduction in the ATPase activity of both proteins. Our data suggest that this interaction may be another component of the AAV-HPV bidirectional interaction.

AAV Rep78 and HPV E1 both are members of the Superfamily III (SF 3) hexameric helicases which includes SV40 large T antigen (*68*, *69*), the DnaB and Rho proteins of E. Coli (*70*, *71*), the gp41 helicase of bacteriophage T4 (*72*) and the gp4A and-B helicase−primase proteins of bacteriophage T7 (*73*). Analytical study (*33*) on the oligomerization domain of AAV Rep78 described two independent domains: (i) NTP-binding domain, and (ii) an α-helical region of a short coiled−coil motif. This study also suggested that the short coiled−coil motif of 30 aa directs the possibility

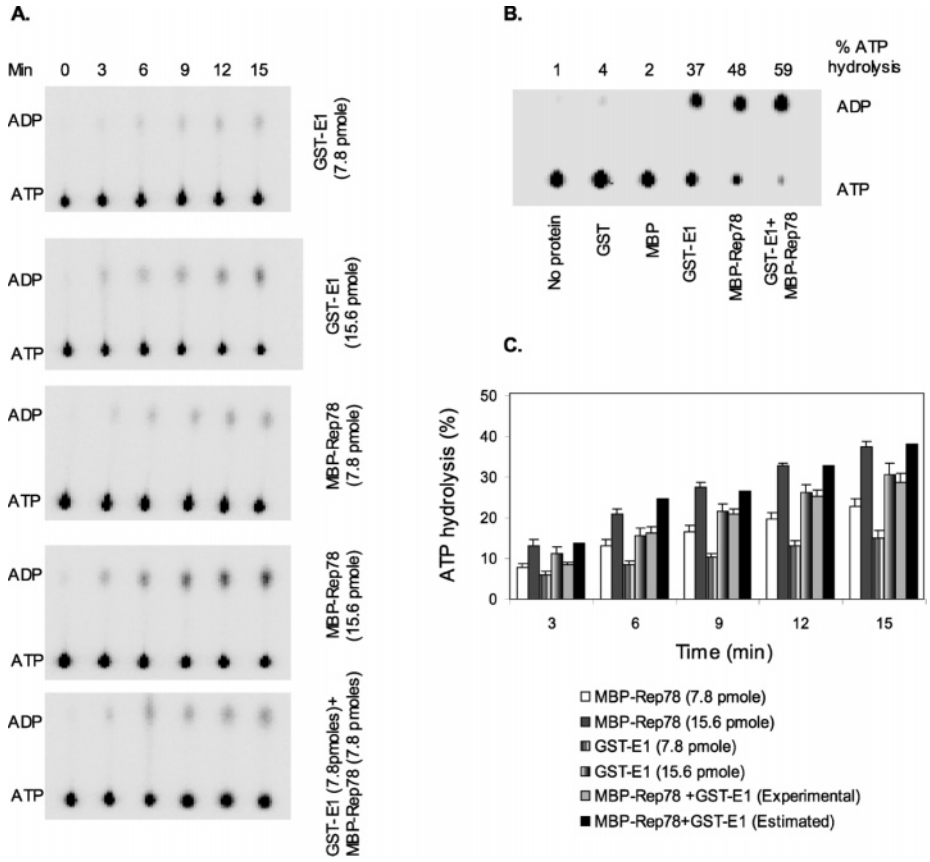

FIGURE 9: Modulation of ATPase activity of GST-E1 and MBP-Rep78. ATPase assays were performed with 0.066 μM [α-^{32}P]ATP, and the percentage of ATP hydrolyzed was quantitated. Each reaction mixture contained 7.8 pmol or 15.6 pmol of GST-E1 or MBP-Rep78 or both proteins together. The reaction mixtures were incubated at 37 °C, and at a 3 min interval, 1 μL of reaction mixture was spotted on a PEI cellulose sheet. The reaction was terminated after 15 min by the addition of EDTA to a final concentration of 20 mM. The cellulose sheets were developed by 1 M formic acid and 0.5 M LiCl, dried, and scanned by a molecular imager (BioRad), and the volume of the radioactive spots was quantitated by Quantity One software. Error bars represent one standard deviation from at least three trials. (A) Represents ATP hydrolysis by GST-E1 or MBP-Rep78 or both the proteins together at different time points. (B) ATP hydrolysis was carried out for 40 min in the presence of equimolar amounts of GST and MBP proteins as controls. (C) Quantitative comparison of ATP hydrolysis by GST-E1 or MBP-Rep78 or both proteins together represented in A.

of combinatorial interaction with heterologous cellular or viral proteins (presumably helicases) bearing a compatible interaction domain. There is a plethora of evidence suggesting physical interaction between Rep78 and other viral proteins like SV40 Large T antigen (*64*) which has similarities with HPV E1 (*74*). Many studies have reported a strong sequence alignment based on the conserved domains of the SF3 helicases (*62*, *63*, *81*). This suggested to us that E1 might interact with Rep78. Our goal was to demonstrate the interaction of Rep78 and E1 by positive-binding assays. As shown in Figures 2 and 3, Rep78 and E1 interaction could be detected by protein affinity pull-down assays and coimmunoprecipitation assay. We also demonstrated that this interaction could be detected by ELISA-based protein interaction assays (Figure 4A,B).

Furthermore, using chemical cross-linking to preserve interactions, it was shown that Rep78−E1 can form hetero-oligomeric complexes *in vitro* (Figures 5, 6, 8). In the presence of BS^3, Rep78 could form several oligomers. It is known that in course of reaction, all Rep78 monomers first take the dimerized form. Therefore, it is likely that the high molecular mass bands correspond to the secondary product of cross-linking of two dimers, i.e., tetramers, and leading to the formation of hexamers. Addition of E1 was demonstrated to produce additional complexes. Rep78 and E1 are known to form homohexamers, required for their activity. It could be speculated that the appearance of an additional band in the presence of both E1 and Rep78 may be an indication of heterohexamer formation, as we identified at least two cross-linked protein bands which appear to contain both Rep78 and E1 (being identified by both anti-GST and anti-MBP antibody) (Figure 7D). But the stoichiometry of E1 and Rep78 molecules in the heterohexameric complexes cannot be predicted at this point because of the near equal size of the two fusion proteins. It is possible that the two proteins are interacting in the form of multiple different heterohexameric forms, of differing stoichiometries. However, at this point the specific forms of Rep78 and E1 multimerization *in vitro* could not be resolved. Here we used a high concentration of BS^3, and proteins were present at high concentration in solution but the *in vivo* situation could be different, where Rep78−E1 multimerization might be promoted by specific trafficking, nuclear-site concentration, other interacting factors, or post-translational modifications. Rep78−E1 cross-linking experiments indicated that the cross-linking was enhanced in the presence of ATP/Mg^{2+}. ATP is required for multimerization of Rep78 (*33*, *20*). Moreover, several studies have reported ATP-dependent increase or stabilization of the oligomerization of other helicases [NS1 of minute virus of mice (*75*), helicase II of *E. coli* (*76*) gp41

and gp4 of helicases of T4 and T7 (*72*, *73*)]. Hexamerization of SV40 T antigen and HPV E1 is also enhanced by NTPs (*77*). So, we speculate that presumed heterohexameric assembly of Rep78 and E1 might also be enhanced by ATP. Binding of ATP may induce a conformational change of either Rep78 or E1 or both which facilitates their heterooligomeric complex formation. There may be some controversy over the use of GST and MBP fusion proteins to analyze the interaction of E1 and Rep78 *in vitro*. But it is well accepted that their activities do not change because of the presence of a MBP or GST moiety, and others/ourselves have taken advantage of the GST and MBP moieties for manipulation with an anti-GST or anti-MBP antibody (*65*, *78*, *79*).

How might Rep78−E1 affect their individual ATPase activity? Both protein's ATPase activities require homomultimers, and both are known to form a hexamer in the presence of ATP. Generally, the ATPase activities of these type of helicases have a sigmoidal dependence on protein concentration indicating that two or more molecules must form a complex to be active (*80*). A major finding of this study was that the ATPase activity of E1 was doubled with twice the concentration of the protein, but the activity of Rep78 was significantly less than double (Figure 9C). The mechanism of the lack of doubling of the ATPase activity of Rep78 upon doubling of the amount of Rep78 is unclear and has never been described before. The higher concentrations of Rep78 protein should be self-promoting (synergistic) for ATPase activity as the formation of ATPase-active Rep78 multimers and high molecular aggregates increasingly favored at protein concentration 100 μg/mL (*81*) (our experimental concentration was 100 and 200 μg/mL). Possibilities of Rep78-self-inhibition include a different requirement of substrate concentration, especially for Mg^{2+}, or the ATP concentration which might not be saturating with respect to the Rep78 concentration. Though, again, we must point out that under the same experimental conditions, the activity of E1 was specifically doubled with the amount of the protein, and no self-inhibition was observed.

Another major finding of this study was that the ATPase activity of E1 and Rep78 was reduced when present together, compared to the summation of their individual ATPase activities. When present together in equimolar amounts, it is possible that (i) their own individual activities are inhibited by the presence of each other, (ii) Rep78 is enhancing E1's ATPase activity while losing its own, (iii) E1 is enhancing Rep78's ATPase activity but its own activity is decreasing, but most probably their individual ATPase activity is reduced in the presence of each other because the interaction of Rep78 and E1 probably affects their ATPase domain, as they have a strong sequence homology in these domains. It is possible that the decreased activity is due to their direct binding, promoting multimerization, or by physically blocking the ATPase domain though the nature of the binding domain is not yet clear. Moreover, it was also possible that, in the presence of each other, there was a molecular rearrangement of E1 or Rep78 oligomeric structure which has reduced ATPase activity. Studies on the structure analysis of HPV E1 revealed that the functional helicase binds to the *ori* as a double hexamer with an intermediate double trimer (*82*). A recent study suggested that the double hexamer consists of two individual hexamers that encircle opposing single strands of the *ori* (*83*). These two hexamers take different configurations for ATP binding and ssDNA binding. ATPase activity of E1 resides in the oligomeric form of E1 (*84*). Formation of heterooligomeric Rep78−E1 complexes may change their configurations so that it could bind to ATP less efficiently, as an overall decrease in ATPase activity was observed in the presence of each other.

The other important functions of Rep78 are helicase activity and nicking activity which require ATP. The specific steps that coupled helicases and ATP hydrolysis required in a replication are still not clear. According to the current model of AAV replication, Rep78 binds its cognate dsDNA-binding site and uses the energy of ATP hydrolysis to either partially unwind the double stranded *ori* sequence or induce the formation of a stem-loop structure, thereby exposing the rep cleavage site in the form of ssDNA (*85*). As the overall ATPase activity of Rep78 is decreased in the presence of E1, at this point, we cannot explain how decreased ATPase activity can be linked to increased AAV replication as demonstrated by our previous studies (*52*, *56*). One possibility might be that the physical interaction of E1-Rep78 might allosterically modulate the structure of Rep78, rendering the protein more active. Alternatively, it may facilitate multimerization and DNA binding, recruit more Rep78 on DNA to form the replication complex, or may stabilize the replication complex as a result of structural change. Yet another possibility is that the presence of AAV's terminal repeat DNA is required to reveal additional biochemical properties of these Rep78−E1 heteromeric multimers. Further study is required to check whether this decreased ATPase activity is, in turn, directly linked to decreased (or increased) HPV or AAV replication. While these data support a physical interaction between Rep78 and E1, they do not exclude the possibility of an indirect interaction between E1 and Rep78 *in vivo*.

What could be the effect of E1-Rep78 interaction in AAV life cycle? For AAV, this interaction could be of critical significance in all aspects of the viral life cycle including productive replication. Regulation of gene expression by Rep78 is complex. Rep78 can act as a repressor as well as an activator of transcription (*22*, *23*). In absence of a helper virus, Rep78 inhibits transcription from its p5 and p19 promoters. In the presence of a helper virus, Rep78 acts as both a repressor and an activator of transcription. The repression of p5 by Rep78 is dependent on site specific DNA binding and consensus ATP binding motif. It is likely that observed Rep78 and E1 interaction decreases the repression by modulating the structure of Rep78. The replication of AAV occurs by a strand displacement mechanism which requires ATP-dependent site-specific endonuclease activity of Rep78 (*21*). It is possible that E1 is enhancing the binding of Rep78 to the DNA, but this speculation does not necessarily explain the higher replication of AAV in the presence of E1 (the helper effect of E1), while putatively decreasing the ATPase activity of Rep78. However, it should be pointed out that while the ATPase activities of Rep78 and E1, when added together, are reduced over their activities alone, the overall ATPase activity is still higher than their individual activity. Perhaps this additional overall ATPase activity contributes to the increased AAV replication in the presence of HPV. Another possibility is that the observed interaction of Rep78 and E1 is linked with interaction to

other cellular proteins, whose overall activity promotes the assembly of Rep78 on ITR. This hypothesis has some appeal, as both proteins are known to interact with multiple cellular proteins and to self-associate so that a lattice of protein−protein interactions is formed.

What could be the affect of Rep78−E1 interaction in HPV life cycle? Several studies have demonstrated that while AAV receives helper function from HPV, high levels of AAV inhibit HPV replication (*56*). Genetic studies showed that AAV Rep78 is responsible for this inhibition, at least in part, by inhibiting p97 promoter of HPV-16 (*86*) or disrupting the binding of TATA-binding protein (TBP) to the TATA box of the p97 core promoter (*31*). But in the 'raft' culture system which supports the productive replication for both HPV and AAV, AAV can both positively and negatively affect HPV replication in an m.o.i.-dependent manner (*56*). Again, this study demonstrates an overall decrease in ATPase activity per molecule when HPV replication protein E1 and AAV replication protein Rep78 are together, and this partially explains the inhibition of HPV replication by AAV. But this may not be the direct consequence of Rep78−E1 interaction. Our laboratory is presently engaged in understanding exactly how Rep78−E1 interaction helps to inhibit HPV replication.

In conclusion, this study demonstrates that Rep78 and E1 physically interact *in vitro*, suggesting a direct mechanism of action for E1 modulating the AAV replication/E1 helper effect. The specific modulation of Rep78's ATPase activity by E1 interaction suggests a specific line of future inquiry into E1's helper activity. Equally exciting are the same issues of Rep78−E1 interaction regarding HPV's life cycle. Additional studies of E1 and Rep78 interaction along with AAV's terminal repeat DNA and analysis of Rep78's additional biochemical activities (DNA recognition and endonuclease activities) are needed to further probe the meaning and importance of E1-Rep78 interaction. Studies on E1's and Rep78's binding domains are also required to further understand the E1-Rep78 interaction. Finally, it was surprising that Rep78 was self-inhibiting in regards to its ATPase activity, under conditions which E1's ATPase activity was not.

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