Mimicry of Cellular A Kinase-Anchoring Proteins Is a Conserved and Critical Function of E1A across Various Human Adenovirus Species

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ABSTRACT The E1A proteins of the various human adenovirus (HAdV) species perform the critical task of converting an infected cell into a setting primed for virus replication. While E1A proteins differ in both sequence and mechanism, the evolutionary pressure on viruses with limited coding capacity ensures that these proteins often have significant overlap in critical functions. HAdV-5 E1A is known to use mimicry to rewire cyclic AMP (cAMP) signaling by decoupling protein kinase A (PKA) from cellular A kinase-anchoring proteins (AKAPs) and utilizing PKA to its own advantage. We show here that E1As from other species of HAdV also possess this viral AKAP (vAKAP) function and examine how they manipulate PKA. E1A from most species of HAdV examined contain a small AKAP-like motif in their N terminus which targets the docking-dimerization domain of PKA as the binding interface for a conserved protein-protein interaction. This motif is also responsible for an E1A-mediated relocalization of PKA regulatory subunits from the cytoplasm into the nucleus, with species-specific E1A proteins having preference for one particular isoform of PKA subunit over another. Importantly, we showed that these newly characterized vAKAPs can integrate into cAMP-responsive transcription as well as contribute to viral genome replication and infectious progeny production for several distinct HAdV species.

IMPORTANCE These data enhance the mechanistic knowledge on how HAdV E1A manipulates cellular PKA to benefit infection. The work establishes that mimicry of AKAPs and subversion of PKA-mediated cAMP signaling are conserved features for numerous human adenoviruses. This study also highlights the molecular determinants conferring selective protein-protein interactions between distinct PKA regulatory subunits and the different E1A proteins of these viruses. Additionally, it further emphasizes the utility of using viral proteins like E1A as tools for studying the molecular biology of cellular regulatory pathways.

KEYWORDS A-kinase anchoring protein, adenovirus, cyclic AMP, E1A, evolution, protein kinase A, protein-protein interaction, replication, viral AKAP, viral mimicry

The E1A oncoprotein of human adenovirus (HAdV) is the first viral protein produced upon infection and performs numerous crucial tasks required for viral command of the host cell (1, 2). By binding to and modulating the activities of important cellular regulatory proteins, E1A alters the transcriptional landscape of infected cells to favor viral replication (3–5). The targets of E1A are diverse in both form and function, highlighting its modular and multifunctional nature. As a relatively small protein, E1A is densely packed, with protein interaction motifs ranging in size from a mere few to dozens of amino acids (2, 6). Additionally, many of these motifs are functional mimics

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of cellular counterparts, suggesting E1A has coevolved with its host to be an effective competitor for its targeted binding partners.

The N terminus of HAdV-5 E1A (amino acids [aa] ~1 to 40) alone is capable of interacting with roughly a dozen cellular proteins and contains strong transcriptional activation ability (2, 7, 8). Indeed, viral genes as well as cellular genes involved in cell cycle, signaling, differentiation, and immunity are modulated by the protein-protein interactions from this small portion of a single viral protein. Recently, protein kinase A (PKA) was shown to be a target of HAdV-5 E1A via viral mimicry (9). PKA primarily exists as a tetrameric holoenzyme consisting of two catalytic subunits (C) bound by a regulatory subunit (R) homodimer (10). Multiple isoforms of both C and R subunits exist, providing additional levels of functional specificity based on cell type-specific expression, additional binding partners, and substrate recognition. C α specifically was manipulated by HAdV-5 E1A via E1A's ability to bind both the RI α - and RII α -type regulatory subunits. This was mediated through the N terminus of HAdV-5 E1A, which contains a binding motif similar to that found in PKA's canonical cellular interacting partner(s), A-kinase anchoring proteins (AKAPs). AKAPs are polyvalent and highly dynamic scaffold proteins that provide spatiotemporal control of PKA by localizing it within the cell (11–13). Their hallmark PKA-binding motif is an amphipathic α -helix that directly binds with high affinity to the hydrophobic groove in the D/D domain of PKA R-subunit dimers. Most AKAP-PKA interactions are specific for either RI α or RII α subunits, but dual-specificity AKAPs exist as well. In addition to binding PKA, AKAPs typically contain motifs directing their subcellular localization to specific intracellular compartments or organelles. AKAPs enable tight and efficient control of cAMP signaling by simultaneously tethering interactions between PKA, substrates, and up- or downstream regulators of the pathway. PKA serves several signaling roles in cell biology, including transcriptional regulation, making it an attractive target for manipulation by a viral hub like E1A (14–17). E1A's own amphipathic α -helix motif allowed it to outcompete endogenous AKAPs to bind with PKA and relocalize it to the nucleus. The alteration of PKA compartmentalization by this viral AKAP (vAKAP) was biologically significant, as it enhanced viral transcription, protein synthesis, and progeny production.

The mechanism behind E1A-mediated manipulation of PKA was well characterized in the context of HAdV-5 infection. However, while protein-protein binding was shown to be largely conserved between PKA and E1A proteins of different HAdV species, very little is known regarding how E1A from other HAdVs may affect this important cellular kinase. The N-terminal sequence of E1A, containing the PKA-binding α -helix, is not as conserved as other portions of this protein (18), and it has been shown that altering a single or a few amino acids can disrupt the interaction between E1A and PKA (9). Whether all species of HAdV utilize E1A as a vAKAP has yet to be determined. There may also exist various preferences for PKA subunit binding or relocalization, and this may confer differential effects on replication for distinct HAdVs.

Here, we analyze representative E1A proteins from seven HAdV species for their abilities to act as vAKAPs. We found that while not all species of E1A could engage in this form of mimicry, the mechanism described for HAdV-5 E1A is largely conserved between several distinct HAdVs. Building upon previous observations, different E1As exhibit a preference for one type of PKA regulatory subunit (RI α or RII α) over the other. While exploring this finding, we identified individual residues in the PKA-binding motifs of vAKAPs that, when altered by site-directed mutagenesis, can convert the preferred interaction to the alternative regulatory subunit. Additionally, we provide new mechanistic insights into how HAdV alters cellular cAMP signaling and show viruses capable of manipulating PKA have an enhanced ability to drive cAMP-regulated transcription. An E1A-PKA interaction is also a conserved requirement for wild-type (WT) levels of viral genome replication and infectious progeny production. Together, our results characterize a shared mechanism for viral manipulation of a cellular kinase which enhances the life cycle of numerous human adenoviruses.

FIG 1 Protein kinase A subunits are conserved binding targets of HAdV E1A's N terminus. HT1080 cells were cotransfected with PKA subunits and various WT or mutant E1A constructs from 7 HAdV types (shown in the inset). Whole-cell lysates were harvested for coimmunoprecipitation. WT E1A from HAdV-3, -5, -9, -12, and -40 associates with RIIα, RIIβ, and Ca, whereas E1A from HAdV-4 and -52 does not. Deletion of the entire E1A N terminus ablated the interactions, whereas the N terminus alone was sufficient for binding.

RESULTS

Protein kinase A subunits are conserved binding targets of the N-terminal region of E1A across numerous HAdV species. The full-length isoforms (13S) of E1A proteins from representative viruses of six HAdV species (A to F) were previously tested for protein-protein interactions with PKA subunits. E1A from HAdV-12 (species A), HAdV-3 (species B), HAdV-5 (species C), HAdV-9 (species D), and HAdV-40 (species F) could associate with PKA via coimmunoprecipitation (co-IP), while HAdV-4 (species E) could not (9). However, the detailed mechanisms behind these individual interactions have only been further explored for HAdV-5 and HAdV-12 (9, 19). Additionally, a seventh species of HAdV (G) has been recently classified, containing a lone member, HAdV-52 (20). To further investigate these E1A-PKA interactions, HT1080 fibrosarcoma cells were transfected with vectors expressing PKA subunits along with either WT E1A from the seven HAdV species or the indicated fragments/portions of E1A (Fig. 1).

Coimmunoprecipitation analysis revealed that RIIα, RIIβ, and Ca all associated with the majority of the E1A proteins tested. E1A from HAdV-5 was used as a positive control, while the nonbinding E1A from HAdV-4 was used as a negative control. E1A from HAdV-4 and the less-characterized HAdV-52 were the only nonbinding types observed. For the E1As which bound (HAdV-3, -5, -9, -12, and -40), the N-terminal portion of these proteins was both necessary and sufficient for conferring these interactions, although this was notably weakest for HAdV-40 E1A. These data show, for the first time, where PKA binds on each of these unique E1As, and the observations align with previous ones for both HAdV-5 and -12 (9, 19). Additionally, this region of E1A has been previously shown to be associated with alterations in cAMP signaling (21–25) and has been characterized as being a conserved transactivation domain (7, 8).

E1A proteins that bind PKA use an AKAP-like sequence motif. The minimal region of E1A required to bind PKA was previously delineated for HAdV-5 (9). To determine the regions within E1A for the other HAdVs whose E1As have putative viral

FIG 2 E1A proteins that bind PKA use an AKAP-like sequence motif. (A to D) HT1080 cells were cotransfected with PKA subunits and E1A constructs from either HAdV-3, -9, -12, or -40. Mutation schemes are shown in the inset. Cell lysates were harvested, and coimmunoprecipitation analysis of each E1A protein revealed that amino acids 11 to 28 for HAdV-3 (A), aa 12 to 29 for HAdV-9 (B), aa 12 to 27, aa 37 to 49 for HAdV-12 (C), and aa 12 to 26 for HAdV-40 (D) were necessary and sufficient for binding PKA subunits.

AKAP activity, we carried out more detailed mutational analyses for each distinct E1A (Fig. 2A to D). Cells were cotransfected with vectors expressing PKA subunits and either WT or the indicated portions of E1A for several HAdV species. For HAdV-3 (Fig. 2A), -9 (Fig. 2B), -12 (Fig. 2C), and -40 (Fig. 2D), overlapping deletions or truncations within the

FIG 3 HAdV species whose E1A proteins bind PKA do so via PKA's docking-dimerization domain. HT1080 cells were cotransfected with WT E1A from 7 species of HAdV along with either both WT PKA regulatory subunits (R) or both subunits lacking their D/D domains (ΔR). Whole-cell lysates were harvested for coimmunoprecipitation. Deletion of the D/D domains in either HA-tagged RII α or MYC-tagged RII α abrogated the interactions with E1A.

N terminus were tested. HAdV-3, -9, and -40 each contained a small region that was both necessary and sufficient for associating with PKA subunits (amino acids 11 to 28, 12 to 29, and 12 to 26, respectively). Each of these regions bears strong similarity to the PKA-binding motif within HAdV-5 E1A, as well as to those of cellular AKAPs (9). Also like HAdV-5 E1A and cellular AKAPs, these motifs are predicted to contain α -helical secondary structure (2). HAdV-12 E1A appeared to have two regions contributing to its binding with PKA (amino acids 12 to 27 and 37 to 49), and deletion of both was required to fully abrogate this interaction. While unexpected, this observation does align with that of a previous report on this E1A's interaction with PKA (19) and suggests additional, unconventional means of manipulating PKA by this E1A type. Taken together, these results demonstrate that the utilization of AKAP mimicry is a conserved function of the E1As which bind to PKA.

E1A binds to PKA via the PKA docking-dimerization domain. Cellular AKAPs bind PKA via the docking-dimerization (D/D) domain of PKA regulatory subunits (RI α and RII α) (11, 12). Given the predicted sequence and structural similarities between cellular AKAPs and the E1As that bind PKA, we tested if the D/D domain was necessary for the observed E1A-PKA interactions (Fig. 3). Cells were cotransfected with WT E1A from seven HAdV species along with either WT PKA regulatory subunits or mutants lacking their respective D/D domains (RI α Δ 1-63 and RII α Δ 1-45). As expected, neither HAdV-4 nor -52 E1A interacted with any PKA construct tested. E1A from HAdV-3, -5, -9, -12, and -40 coimmunoprecipitated with WT PKA regulatory subunits but failed to associate with those lacking the D/D domains. Thus, multiple E1As not only resemble AKAPs themselves but also require the same site on the PKA regulatory subunits that is targeted by cellular AKAPs and the previously characterized HAdV-5 E1A for interaction.

Distinct E1A proteins relocalize PKA regulatory subunits in a type-specific manner. The subcellular redistribution of PKA subunits was previously explored with E1A from HAdV-5 and HAdV-12. Those two proteins were found to have contrasting preferences for the type I and type II regulatory subunits, respectively (9, 19). Here, we sought to extend our investigation into how E1A proteins from different HAdVs affect PKA by utilizing both infection- and transfection-based approaches. HeLa cells were transiently transfected with enhanced green fluorescent protein (EGFP)-tagged WT E1A proteins from all seven HAdV species, and proteins were subsequently detected via autofluorescence (EGFP) or immunofluorescence staining (PKA) (Fig. 4A to C). While PKA C α appeared nuclear-cytoplasmic and broadly similar in all immunofluorescence experiments (data not shown), notable differences emerged between RI α and RII α . As

FIG 4 Distinct E1A proteins relocalize PKA regulatory subunits in a type-specific manner. HeLa cells were transfected with EGFP-tagged WT or mutant E1A constructs from 7 species of HAdV. Cells were fixed and permeabilized, and proteins were detected via autofluorescence (E1A) or by immunofluorescence (PKA). Scale bars represent 100 μ m, and the targeted PKA subunit is indicated above each group of panels. (A) EGFP alone or E1A from HAdV-4, -52, and -40 showed no relocalization of either PKA regulatory subunit. E1A from HAdV-3 and -12 showed no relocalization of R1 α , whereas HAdV-5 and -9 E1A showed no relocalization of R2 α . (B) HAdV-5 and -9 E1A caused a shift of R1 α from the cytoplasm to the nucleus that was ablated when these E1A proteins had their AKAP-like motifs deleted. (C) Conversely, HAdV-3 and -12 E1A caused a shift of R2 α from the cytoplasm to the nucleus that was ablated when their PKA-binding regions were removed. Quantification of nuclear signal relative to total cellular signal is also shown next to representative images for the indicated E1A species (values are displayed as means $\pm$ standard deviations; *, $P < 0.001$; $n = 50$).

FIG 5 C terminus of E1A, which contains the nuclear localization signal, is required for efficient retasking of PKA. A549 cells were infected with either HAdV-5 *d/520* or *d/1131/520* (the E1A proteins expressed by these viruses are shown in the adjacent panel). After 24 h, cells were fixed, permeabilized, and stained for confocal immunofluorescence. E1A lacking the C-terminal bipartite NLS was deficient for both its nuclear localization and re compartmentalization of Rl α . Scale bars represent 100 μ m.

expected, neither HAdV-4 nor HAdV-52 E1A, which did not bind PKA, showed any ability to alter the subcellular localization of PKA subunit Rl α or Rll α (Fig. 4A). The relocalization phenotypes for both HAdV-5 (Rl α specific) and HAdV-12 E1A (Rll α specific) were reproduced as positive controls. This confirmed that different E1As exhibit a type preference for functionally altering the PKA subunits they bind (Fig. 4B and C). Similar to HAdV-5, E1A from HAdV-9 caused the Rl α subunit to be redirected from the cytoplasm into the nucleus (Fig. 4B). In contrast, E1A from HAdV-3 caused the Rll α subunit to be relocalized, similar to E1A from HAdV-12 (Fig. 4C). HAdV-40 E1A did not appear to relocalize either PKA regulatory subunit (Fig. 4A).

Mutant E1As containing deletions which abrogate PKA binding for each species were tested side by side with the cognate WT E1A control (Fig. 4A to C). As expected, the removal of the previously established binding motifs within each E1A protein abrogated the protein's ability to relocalize PKA under transfection conditions. The relocalization of PKA regulatory subunits was therefore E1A dependent and required the AKAP-like motif necessary for PKA interaction, as previously demonstrated with HAdV-5 E1A. Using HAdV-5 as a model, we also demonstrated that the nuclear localization signal (NLS) of E1A, but not CR3, is needed to contribute to PKA relocalization (Fig. 5). While WT 12S virus (*d/520*) relocalized Rl α despite the absence of CR3, a mutant lacking both CR3 and the C-terminal bipartite NLS (*d/1131/520*) was deficient for relocalization of Rl α . This suggests that binding of the regulatory subunit by E1A is not sufficient for efficient re compartmentalization of PKA and that relocalization requires cooperation with other functions located in the C terminus of E1A. PKA localization was also examined in A549 cells infected with WT strains of HAdV-3, -4, -9, and -12 (Fig. 6). The observed staining phenotype matched that of the transfection-based experiments, suggesting that *in vitro* results faithfully reflect what occurs during an *in vivo* infection.

The curious observation that E1A proteins are able to associate with both Rl α and Rll α in co-IP experiments yet only relocalize one isoform *in vivo* led us to test whether differences in binding affinity for distinct subunits contributed to this selectivity. First, we established a co-IP-based squelching assay where untagged HAdV-5 E1A was titrated as a competitor against EGFP-tagged HAdV-5 E1A (Fig. 7A). As expected, higher levels of competition eventually abrogated the ability of EGFP-E1A to coimmunoprecipitate with PKA regulatory subunits. We then applied these conditions to similarly test HAdV-5 E1A against EGFP-tagged E1A from HAdV-3, -9, -12, and -40 (Fig. 7B). HAdV-3 and -12 E1As were more resistant to competition from HAdV-5 E1A when examining their associations with Rll α ; conversely, their interactions with Rl α were more susceptible to depletion in this competition assay. Together these data suggest again that HAdV-3 and -12 have relatively higher affinity for Rll α while HAdV-5 prefers Rl α . HAdV-9 E1A was susceptible to competition in a manner similar to how HAdV-5 was against

FIG 6 Localization of PKA regulatory subunits in cells infected with various HAdV species. A549 cells were infected with WT HAdV-3, -4, -5 (*d/309*), -9, or -12 at an MOI of 5 (for HAdV-5) or 25 (for all other viruses). After 24 h cells were fixed, permeabilized, and stained for confocal immunofluorescence. R1α appears to be more nuclear in HAdV-5- or -9-infected cells than in uninfected cells. In contrast, R11α appears more nuclear in HAdV-3- or -12-infected cells. Scale bars represent 100 μ m.

itself (Fig. 7A), reinforcing the similarity between these two E1A species. HAdV-40 E1A was the most susceptible to competition of the E1A proteins, indicating that its association with PKA is the weakest.

While E1As clearly have a preference to relocate and bind one specific PKA isoform, the ability to functionally affect the other isoform remains a possibility. Using HAdV-5 as a model, we infected A549 cells pretreated with either a scrambled control short interfering RNA (siRNA) or siRNA knocking down R1α, HAdV-5 E1A's preferred regulatory subunit, with WT (*d/309*) virus (Fig. 8A). In control cells, HAdV-5 E1A behaved as expected by relocating R1α into the nucleus while leaving R11α in the cytoplasm. However, when cells were deprived of R1α protein, some R11α was localized within the nucleus along with E1A, a finding observed previously only for HAdV-3 and -12 E1A. This suggests the siRNA-mediated removal of the R1α isoform of PKA shifted the binding equilibrium of E1A with R11α *in vivo*, and that the increased interaction was sufficient to allow some of this isoform to be relocated (Fig. 8B). While the observed recompartmentalization was not quite as dramatic as that with R1α, this may be due to HAdV-5 E1A's apparent lower affinity for this particular subunit.

E1A's ability to act as a vAKAP is modular and malleable by mutagenesis. To test if the viral AKAP function of E1A was modular, we created a set of EGFP-tagged constructs that combined various portions of E1A from HAdV-4 and HAdV-5 to form chimeric proteins. Specifically, the N-terminal domains of each protein were swapped for the analogous region in the other. When these constructs were tested alongside WT

FIG 7 Different E1A species display an affinity preference for either R1α or R11α. (A) HT1080 cells were cotransfected with EGFP-tagged HAdV-5 E1A and PKA regulatory subunits. Untagged HAdV-5 E1A was titrated as a competitor against EGFP-E1A for binding PKA. Whole-cell lysates were harvested for co-IP using an antibody against EGFP. Increasing amounts of untagged E1A squelched EGFP-E1A's interactions with R1α and R11α. (B) Conditions optimized from panel A were used in a larger competition panel with EGFP-tagged E1A from HAdV-3, -9, -12, and -40. HT1080 cells were similarly cotransfected with the indicated E1A species, PKA subunits, and increasing amounts of untagged HAdV-5 E1A. Co-IP analysis revealed various resistances and susceptibilities to competition from HAdV-5 E1A for binding either R1α or R11α.

counterparts for binding PKA subunits via co-IP, the chimera with the HAdV-5 N terminus and HAdV-4 C terminus gained the protein-protein interactions exhibited by WT HAdV-5 E1A, whereas the reciprocal chimera lost them (Fig. 9A). Subsequently, these chimeras were transfected into HeLa cells and PKA subcellular localization was examined via immunofluorescence staining (Fig. 9B). As with the co-IP assays, the HAdV-4 chimera gained the ability to relocate the PKA R1α subunit when its N terminus was replaced with that from HAdV-5. In contrast, the HAdV-5 chimera with the HAdV-4 N terminus behaved like the EGFP negative control and WT HAdV-4 E1A.

To explore this concept of modularity further, we aimed to test if swapping individual residues within the AKAP-like motif of different E1As could alter their preference for relocating one type of PKA subunit over the other. By comparing the sequences of the AKAP-like motifs between HAdV-3 and HAdV-5 E1A, triple point mutants for both E1As were designed (HAdV-5 D21E, E26T, V27L and HAdV-3 E22D,

FIG 8 Depriving HAdV-5 E1A of type I PKA causes E1A to retask type II PKA instead. (A) A549 cells were treated with the indicated control or R1α-specific siRNAs and subsequently infected with WT (d309) HAdV-5. Only in the absence of R1α protein does E1A relocate some R11α to the nucleus. Scale bars represent 100 μm. (B) Equilibrium models of how E1A may compete against cellular AKAPs (cAKAP) and determine which PKA isoform it relocates to the nucleus.

FIG 9 Viral AKAP mimicry is a modular function of E1A. (A) HT1080 cells were cotransfected with PKA subunits and either WT or chimeric E1A constructs, and cell lysates were harvested for co-IP. (Inset) Construction of the HAdV-4 and -5 E1A chimeras (the arrow indicates where N-terminal swap occurred). The HAdV-4 chimera gained the ability to bind PKA subunits, whereas this was lost for the HAdV-5 chimera. (B) HeLa cells were transfected with the indicated EGFP-tagged E1A constructs. Cells were fixed and permeabilized, and proteins were detected via autofluorescence (E1A) or by immunofluorescence (PKA). Cells transfected with WT HAdV-5 or the HAdV-4 chimera exhibited a shift of RIα from the cytoplasm into the nucleus. In contrast, WT HAdV-4 or the HAdV-5 chimera showed no noticeable relocalization of RIα. Scale bars represent 100 μ m.

T27E, L28V) in an effort to exchange specificity. These relatively conservative mutations are embedded within the predicted α -helix portions of both proteins and are at analogous positions that were previously demonstrated to be crucial for HAdV-5 E1A binding (9). When tested alongside WT E1A counterparts for binding via co-IP, both mutants retained the interaction with PKA subunits, similar to that of WT E1A (Fig. 10A). The mutants next were transfected into HeLa cells and examined for their ability to relocalize individual PKA subunits (Fig. 10B). Both triple mutants experienced a change in preference for which PKA subunit they relocalized to the nucleus. When mutated in this way, HAdV-5 E1A, which previously preferred RIα, now altered RIIα distribution like WT E1A from HAdV-3 and -12. When HAdV-3 E1A, which normally preferred RIIα, was mutated in this fashion, it relocalized RIα like WT E1A from HAdV-5 or -9. Thus, it appeared that the preference of E1As for specific PKA subunits can be altered using targeted mutagenesis of select residues.

Interaction with PKA is required for full E1A-mediated transactivation of a PKA-driven promoter. It was previously shown that the interaction between E1A and PKA could serve to enhance viral gene expression during HAdV-5 infection (9). Additionally, cotransfected HAdV-12 E1A and PKA could activate the HAdV-12 E2 promoter (19). However, many cellular genes are also known to be regulated by PKA via signaling through cAMP (14–17). Also, E1A has been demonstrated to affect cAMP-responsive cellular genes in certain contexts (24, 26, 27). We aimed to determine if the E1A proteins

FIG 10 Specific residues in the AKAP-like motif of E1As induce preference for specific PKA subunits. (A) HT1080 cells were cotransfected with PKA subunits and the indicated E1A constructs, and cell lysates were harvested for co-IP. Both WT HAdV-3 and -5, along with mutants (HAdV-5 D21E, E26T, V27L and HAdV-3 E22D, T27E, L28V), retain similar levels of binding to PKA. The mutation scheme in the inset shows residue positions previously demonstrated as crucial in HAdV-5, along with the analogous positions in HAdV-3. (B) HeLa cells were transfected with the indicated EGFP-tagged E1A constructs. Cells were fixed and permeabilized, and proteins were detected via autofluorescence (E1A) or by immunofluorescence (PKA). Cells transfected with WT HAdV-3 or the HAdV-5 D21E, E26T, V27L mutant exhibited a shift of RIIα from the cytoplasm into the nucleus. In contrast, WT HAdV-5 or the HAdV-3 E22D, T27E, L28V mutant exhibited a relocalization of RIα. Scale bars represent 100 μ m.

from different HAdVs could insert themselves into this signaling pathway and alter PKA/cAMP-regulated transactivation. HT1080 cells were cotransfected with a luciferase reporter demonstrated to be cAMP and PKA regulated (driven by the rat phosphoenolpyruvate carboxykinase [PEPCK] promoter) along with various E1A constructs (Fig. 11). Initial experiments confirmed that this reporter is both cAMP inducible via forskolin treatment (an adenyl cyclase activator that increases intracellular levels of cAMP) as well as dependent on PKA protein, as shown by siRNA-mediated knockdown. E1As shown to bind PKA were then compared to a species-specific deletion mutant lacking the PKA binding site for their effects on PEPCK-dependent transcription. Although all seven species of E1A were able to transactivate this reporter to levels higher than those of EGFP (vector control) alone, those previously shown to both bind and relocalize PKA functioned as the strongest transactivators (HAdV-3, -5, -9, and -12). Deletion of the AKAP-like PKA-binding motifs in these E1As reduced their ability to drive expression of this reporter construct severalfold. Transactivation by HAdV-40 E1A was weaker than

FIG 11 Interaction with PKA is required for full E1A-mediated transactivation of a PKA-driven promoter. HT1080 cells were transfected with a reporter consisting of the luciferase gene driven by the rat PEPCK promoter (sequence shown in the inset) along with various WT or mutant E1A constructs from 7 HAdV species. A 6-h treatment of 0.5 μM forskolin (fsk) and siRNA-mediated PKA knockdowns was performed to demonstrate this reporter's cAMP responsiveness and PKA-dependent regulation. Protein levels of each E1A construct are also shown. Luciferase activity was measured for each condition, and light units were set relative to an empty plasmid control. Each of the 7 WT E1As tested could transactivate this reporter, with those shown to bind PKA having the strongest effect. For the 5 species that interacted with PKA, deletion of their PKA-binding motifs conferred a statistically significant reduction on E1A's ability to induce this reporter (with the exception of HAdV-40). Values are displayed as means ± SEM. *, $P < 0.05$; $n = 3$.

that of the other E1As, and deletion of its putative PKA-binding region did not affect this function. Thus, similar to the negative result in the relocalization assays (Fig. 4A), HAdV-40 appears to bind PKA in the co-IP assays, but this interaction does not appear to functionally impact PKA-dependent transcription *in vivo*.

HAdV infection stimulates the cellular cAMP pathway. Using HAdV-5 as a model, we further investigated how HAdV infection and E1A expression may cooperate with cellular cAMP signaling. A549 cells infected with either the WT (d/309) or ΔE1A (d/312) or E1AΔ4-25 (d/1101) mutant all showed increased levels of cellular cAMP compared to those of uninfected cells, as visualized by immunofluorescence (WT virus is shown as a representative) (Fig. 12A). This was accompanied by higher levels of phosphorylated CREB1 on Ser133 (a known PKA substrate) (Fig. 12B and C) and higher levels of PKA

FIG 12 HAdV-mediated stimulation of cAMP signaling allows WT E1A to retarget PKA activity. A549 cells were infected with either WT (*d/309*), Δ E1A (*d/312*), or E1A Δ 4-25 (*d/1101*) HAdV-5 (MOI, 5) and examined at the indicated time points postinfection using confocal immunofluorescence (A and B), Western blotting (C), or kinase assay (D). (A) Cellular cAMP levels were examined before and after the onset of viral replication (as indicated by staining for HAdV-5 ssDNA-binding protein [DBP]). Production of cAMP is noticeably higher than that of uninfected cells both before and after E1A is expressed. Results with WT virus are shown as representative of all infected conditions. (B) Increased levels of phosphorylated CREB1 on Ser133 are present relative to uninfected cells and are shown for WT virus, localizing adjacent to and overlapping with virus replication centers. Scale bars represent 100 μ m. (C) Western blot analysis of phosphorylated CREB1 detected increased levels under all infection conditions, which was highest in WT-infected cells after production of E1A protein. (D) Cell lysates were measured for PKA kinase activity and displayed similarly higher levels of activity under all infection conditions. Values are displayed as means $\pm$ SEM. *, $P < 0.05$; $n = 3$. (E) HT1080 cells were cotransfected with PKA regulatory and catalytic subunits along with HAdV-5 E1A. Co-IP analysis showed that E1A does not dissociate the PKA holoenzyme. (F) HT1080 cells were cotransfected with R1 α and/or R11 α in the presence or absence of the HAdV-5 E1A N terminus and subjected to co-IP. No heterodimerization of PKA regulatory subunits was detectable even with a long-exposure (LE) Western blot.

kinase activity (Fig. 12D). Each of these findings was examined at time points before and after the onset of E1A protein production. Notably, the levels of PKA kinase activity and cAMP were broadly similar across all time points postinfection for all viruses, indicating that they are independent of E1A and more likely a biological response to

FIG 13 Requirement of PKA for viral replication is conserved across numerous HAdV species. A549 cells were treated with control siRNA or siRNA specific for PKA subunits and infected with HAdV-5 (MOI, 5) or HAdV-3, -4, -9, or -12 (MOI, 25). Values for each virus were normalized to 1.0 in control-treated cells and are displayed as means $\pm$ SEM. $n = 3$. (A) DNA was isolated at 6 h postinfection as a gauge of viral input and at 48 h postinfection to measure viral genome replication. Relative viral DNA levels were quantified by qPCRs using a forward primer that recognizes a conserved sequence in E1A in combination with a species-specific reverse primer. (B) Cells were collected at 72 h postinfection, and production of infectious progeny virus was quantitatively assayed by plaque formation on HEK293 cells. For both genome replication and progeny production, knockdown of PKA regulatory subunits caused impairment for HAdV-3, -5, -9, and -12. The non-PKA binding HAdV-4 was unaffected. Knockdown of either the catalytic subunit or all 3 PKA components together impaired all viruses. *, $P < 0.05$; $n = 3$.

the infection itself. We also showed that E1A itself is not causing aberrant interactions between PKA subunits (Fig. 12E and F). The coexpression of E1A did not inhibit the association between regulatory and catalytic PKA components (consistent with what we know of where E1A and PKA interact with each other), and its N terminus did not induce any noticeable heterodimerization of type I and II regulatory subunits. The most striking observation from these experiments was that only in WT-infected cells and after the onset of E1A protein expression did we observe the highest levels of phosphorylated CREB1 on Ser133. Given that the cAMP and kinase activity levels are similar between cells expressing either WT E1A or a mutant unable to bind PKA, it is likely the increased phosphorylation of this PKA-targeted transcription factor is due to WT E1A's vAKAP function. Under these conditions, high levels of phosphorylated CREB1 were localized immediately adjacent to and overlapping virus replication centers (Fig. 12B).

Requirement of PKA for viral replication is conserved across numerous HAdV species. To firmly establish the biological significance of these distinct E1As as vAKAPs, we assessed PKA's role in multiple steps of the viral replicative cycle. A549 cells were treated with either control siRNA or siRNA specific for the indicated PKA subunits and infected with WT HAdV-3, -4, -9, -12 (multiplicity of infection [MOI] of 25), or -5 (MOI of 5) (Fig. 13A and B). The efficiency of viral genome replication was assessed by

quantitative PCR (qPCR) of genomic viral DNA at 48 h postinfection, compared to an input sample at 6 h postinfection, and normalized to cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fig. 13A). For HAdV-3, -5, -9, and -12, knockdown of the PKA regulatory subunits resulted in an inhibition of viral DNA replication. This was not observed for HAdV-4, whose E1A does not bind PKA. This suggests that viruses that bind and relocalize PKA benefit from the interaction and require it for full, WT levels of DNA replication. Knockdown of the catalytic subunit of PKA resulted in an inhibition for all viruses, including HAdV-4. This may be due to an additional role for this protein in the HAdV replicative cycle that is C α specific and E1A independent.

Production of infectious progeny virus was assayed at 72 h postinfection by plaque assay (Fig. 13B). The production of WT progeny for each HAdV serotype tested mirrored the observed phenotypes for genome replication. Only HAdV-4 was unaffected by the knockdown of PKA regulatory subunits, confirming that viruses that target PKA via E1A do so as part of an important aspect of their replicative cycles. As observed for viral genome replication, knocking down both the regulatory and catalytic subunits in combination did affect all HAdVs tested. This finding matches previous observations for HAdV-5 (28, 29) and again suggests the influence of an alternative, E1A-independent role for the C α protein in HAdV biology.

DISCUSSION

Mimicry is an often-exploited strategy utilized by numerous protein products of HAdV to facilitate virus-host interactions (9, 30–32). HAdV E1A in particular is a remarkably well-connected viral hub protein capable of altering the protein interaction network in an infected cell to create an environment conducive to viral replication (1, 2). By embedding itself deeply within numerous signaling pathways, E1A can exert tremendous influence upon cellular machinery in order to enhance various aspects of the HAdV life cycle. We previously demonstrated that HAdV-5 E1A uses mimicry to act as a viral AKAP, facilitating an interaction with PKA that increased viral transcription, protein synthesis, and progeny production (9). In the present work, we have determined that despite some mechanistic flexibility, the manipulation of PKA via E1A is a conserved and important function for numerous, distinct species of HAdV.

Even with having substantial differences in primary sequence, E1A proteins are often remarkably consistent in their functional similarities (33). Our findings establish that 5 representative members of the 7 species of HAdV express E1A proteins that make protein-protein interactions with PKA. This apparent conservation suggests that the E1A-PKA interaction has played a potentially important role in the evolutionary history of the virus. Indeed, the two HAdVs which do not bind PKA via E1A (HAdV-4 from species E and HAdV-52 from species G), along with HAdV-40 from species F (which bound with lower affinity than other species and did not functionally affect PKA) are considered more closely related to simian adenoviruses than the HAdVs that have E1A proteins that demonstrably function as viral AKAPs (HAdV-3, -5, -9, and -12) (20, 34). Along with lacking vAKAP functionality, these non-PKA-binding E1As also have more dramatic differences in both primary sequence and predicted secondary structure in the region responsible for the interaction than the other HAdV types (2). In particular, HAdV-52 E1A contains a single-amino-acid insertion in the core of this motif that likely distorts the amphipathic nature of the predicted helix. Among primate species, PKA is a well-conserved kinase (10), and it is unknown if closely related simian adenovirus E1A proteins are capable of interacting with simian PKA. However, we speculate that an interaction is unlikely given the lack of the requisite predicted amphipathic α -helical structure in the E1A N terminus of these viruses. Therefore, the capability of some HAdV E1As to function as vAKAPs likely arose from their extended coevolution with humans as a result of providing a selective benefit for the virus within this particular host.

For all viruses that did bind PKA, the N terminus of E1A alone is both necessary and sufficient for this interaction, as previously observed within HAdV-5. These E1As also

target the D/D domains of PKA regulatory subunits as the binding interface in the same manner as cellular AKAPs (11, 12). Furthermore, an AKAP-like motif in the E1A N terminus containing a putative α -helical structure is responsible or at least contributory to this interaction. E1A proteins from HAdV-3, -5, and -9 all contain this motif, which strongly resembles the PKA-binding region of cellular AKAPs. This similarity indicates strong mechanistic conservation. HAdV-12 E1A also contains such a motif but also utilizes a second, downstream region contributing to this E1A-PKA interaction. This finding confirms the previously unexplored observation that HAdV-12 E1A bound PKA via two different N-terminal regions (19). While recent studies of cellular AKAPs have revealed the ever-increasing diversity of PKA-binding motifs (35), this secondary region bears no obvious similarity to the canonical α -helical domain utilized by cellular AKAPs. It is possible that the largely unstructured nature of E1A allows for a second, novel binding site to form in solution, which strengthens PKA binding, a feature observed with other targets of E1A (6). This finding also reveals that viral protein sequences outside canonical AKAP mimics may be capable of regulating PKA under both normal and infection conditions.

During infection, multiple HAdV species induced a subset of PKA regulatory subunits to relocate from the cytoplasm to the nucleus. Transfection-based experiments using a range of E1A deletion mutants for each HAdV species confirmed that this phenotype was dependent on the PKA-binding motifs of E1A. Different E1As had differing effects on specific PKA regulatory subunit types. While E1A from HAdV-3, -5, -9, -12, and -40 all could bind PKA in separate co-IP experiments, HAdV-3 and -12 E1A specifically relocalized the RII α subunit, whereas HAdV-5 and -9 E1A preferred the RI α subunit. HAdV-40 E1A does not display any ability to relocalize PKA, suggesting that the interaction is not functionally relevant *in vivo*. The relative differences in binding affinities between E1A species and PKA isoforms was confirmed in competition assays, reinforcing the idea of type preference. These data are consistent with previous reports on E1A-PKA interactions for HAdV-5 and -12 (9, 19), and a type preference is also a feature of many cellular AKAPs (13). This is also the first report of both HAdV-3 and -9 E1A acting as viral AKAPs and explores the molecular determinants of this conserved E1A function in the greatest detail to date.

Our established viral AKAP model suggests that E1A uses the direct interaction with PKA regulatory subunits as a bridge to associate with the catalytic subunit, C α (9). C α was previously shown to be recruited to the promoters of E1A-regulated viral genes and was capable of enhancing transcription from these sites (9). It is unclear if the preference for either RI α or RII α confers the same or different benefits, and this could also be context dependent based on cell- or tissue-specific expression levels. The ability to target both regulatory types may also serve as an evolutionary redundancy, ensuring the crucial interaction with PKA's catalytic component regardless of the availability of individual PKA subunits. To explore this, we deprived cells of RI α protein using siRNA and then infected them with HAdV-5. Under these conditions, where the preferred isoform for HAdV-5 is absent, some relocalization of RII α occurred. Although the degree of relocalization was reduced, this may be related to HAdV-5's relatively lower binding affinity for RII α . Nevertheless, this result suggests that the potential to bind both type I and II PKA to at least some extent is advantageous to HAdV as they may, to a degree, compensate for each other.

While the hydrophobic component of the PKA-binding amphipathic α -helix is a necessary feature in both cellular and viral AKAPs (9, 13), adjacent residues confer critical electrostatic interactions and contribute to the preference for either RI α or RII α (35). We explored this feature in the context of HAdV E1A by successfully creating mutants in HAdV-3 and -5 E1A that reversed each protein's preference for the different PKA regulatory subunits. Using previously validated residue positions in HAdV-5 E1A that are known to affect PKA-E1A interaction (9), triple point mutants were created in both proteins that exchanged these residues in their analogous positions. Both the novel HAdV-3 and -5 mutants retained binding to both types of PKA subunits in co-IP

experiments, but their abilities to relocalize RI α and RII α were reversed. This demonstrates that like cellular AKAPs, individual amino acids in viral AKAPs specify their differential affinities for distinct PKA subunits, enhancing our knowledge of how type I, type II, and dual-specificity AKAPs function.

The targeting of PKA by E1A was previously shown to drive transcription of HAdV-5 early genes via recruitment of C α to viral promoters (9). E1A has also been shown to synergize with cAMP (21–25). Here, we sought to expand upon this mechanism by exploring E1A-PKA interactions for various HAdV species in the context of the cAMP signaling pathway. A luciferase reporter driven by the previously characterized rat PEPCCK promoter (36) was constructed and validated to be both cAMP inducible and PKA dependent. E1A proteins from all 7 species of HAdV could activate expression from this reporter, which was expected given E1A has multiple transactivation domains and mechanisms for inducing transcription (2, 7, 8, 37–39). The E1A proteins from viruses that could bind and relocalize PKA subunits (HAdV-3, -5, -9, -12) induced this reporter to the greatest extent. Deletion of the PKA-binding regions of these E1As severely handicapped their ability to transactivate this construct. Residual activation by these mutants was comparable to the levels observed for WT E1A species unable to bind PKA. This confirms that the PKA interaction contributes to E1A-mediated transactivation of this PKA-responsive reporter.

During HAdV-5 infection, notable alterations to the cellular cAMP signaling pathway occurred. Indeed, increased levels of cAMP relative to uninfected cells were observed by immunofluorescence, as were higher levels of PKA kinase activity and phosphorylation of CREB1 on Ser133. Most of these observations were consistent across time points before and after the production of E1A protein as well as between different HAdV-5 mutants (WT, Δ E1A, and a mutant incapable of binding PKA). This indicates that the stimulation of cAMP signaling is in response to the HAdV infection and independent of E1A. The notable exception to this trend was that in the presence of WT E1A protein, a far higher level of phosphorylated CREB1 was observed both by Western blotting and immunofluorescence, where it localized adjacent to and overlapping virus replication centers. These findings are consistent with our previously established vAKAP model, suggesting that E1A exerts its effects on the cAMP pathway not through broad changes in 2nd messenger levels or PKA catalytic activity but rather via the localized retasking of PKA to sites of action. While HAdV infection may provoke cAMP signaling, a functional vAKAP is needed to fully take advantage of these conditions. Notably, all E1A proteins tested also bind CBP, an important coregulator of CREB via an overlapping region of the N terminus and/or CR3, providing an additional connection between E1A and cAMP-regulated transcriptional control (40–42).

Altogether, these data suggest that E1A proteins can integrate into the cAMP signaling pathway to drive higher levels of cAMP-modulated gene expression, presumably in a localized fashion. This also suggests the exciting possibility that E1A is capable of differentially regulating the hundreds of cellular cAMP-responsive genes during infection. This mechanism could contribute to enhancing the infectious cycle for HAdVs that have a functional E1A-PKA interaction or contribute to the different tropisms and disease pathogenesis of distinct HAdV species.

The conserved importance of PKA during infection was established by examining viral genome replication and progeny production in the context of siRNA-mediated PKA knockdowns for multiple HAdV types. For HAdV-4, which does not bind PKA, knockdown of RI α and RII α did not notably affect either viral DNA replication or progeny virion production. For HAdV-3, -5, -9, and -12, which all have viral AKAPs, the loss of PKA regulatory subunits as targets for E1A reduced the efficiency and productivity of their replicative cycles. As previously established for HAdV-5, the E1A-PKA interaction serves to drive viral gene expression of numerous HAdV genes, including E2, E3, and E4 (9). Many transcripts from these genes encode crucial protein products involved in viral genome replication, transcription, cell cycle modulation, and virus-host interactions (43, 44). Presumably, the shortfall of these critical viral products contributes to the observed reduction in infectious progeny virus. Loss of C α or all PKA subunits in

combination negatively affected all viruses tested, including HAdV-4, which expresses an E1A that does not target PKA. This result suggests an additional, E1A-independent role for $C\alpha$ in HAdV infection. $C\alpha$ activity was previously shown to be crucial for transport of species C HAdV virions to the nucleus during the establishment of infection (28, 29). Our results suggest other species of HAdV also use this process to kick-start this early stage of their replicative cycles. The conserved targeting of multiple different PKA subunits by various viral components at distinct stages of infection underscores the true importance of this cellular kinase for HAdV.

E1A has been repeatedly shown to be adept at influencing various cellular scaffold and multiprotein complexes (9, 45, 46). To date, E1As from various HAdV species are currently the only well-characterized viral AKAPs; however, given the well-connected nature of cellular scaffolds like AKAPs, mimicry and manipulation of similar proteins is a strategy exploited by numerous viral pathogens (47, 48). This allows viruses to control macromolecular networks or nucleate protein-protein interactions to facilitate their own needs (49). By studying how viral regulatory proteins integrate into cellular signaling by mimicking scaffolds, we gain comprehension of how viruses overcome the daunting challenge of reorganizing their hosts into environments conducive for replication. Examining protein-protein interactions between viral and host factors also offers clinically relevant insights. For instance, the manipulation of orthologous cellular components within distinct host species (such as PKA) by viral proteins can contribute to successful cross-species transmission events. By understanding these events, we learn more about disease tropism or severity. Also, studying the molecular basis of these virus-host interactions provides an opportunity to intervene for druggable targets, such as with the E1A-CDK9 interaction for various HAdVs (50).

In summary, we conclusively identified that E1A proteins from multiple species of HAdV can function as viral AKAPs. We also confirmed that they structurally and mechanistically resemble each other, although some flexibility exists, presumably as a result of different evolutionary histories. We have also advanced the understanding of the functional mechanism of these vAKAPs by demonstrating their ability to integrate into cAMP signaling-dependent transcriptional activation. Furthermore, we demonstrate that vAKAPs play an important, biologically significant role in enhancing genome replication and progeny production for multiple HAdV species.

MATERIALS AND METHODS

Cell lines and transfections. Human A549 (provided by Russ Wheeler, Molecular Pathology/Genetics London Health Sciences Centre), HEK293, HT1080, and HeLa (purchased from the American Type Culture Collection) cells were grown at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Multicell Technologies) supplemented with 10% fetal bovine serum (Gibco). Plasmids were transfected into HT1080 and HeLa cells using XtremegeneHP (Roche) by following the manufacturer's recommendations. After 24 h of incubation, transfected cells were used for downstream experiments.

Viruses and infection of cells. Wild-type (WT) HAdV-5 (*d/309*) was previously described (51), and mutants *d/312* and *d/1101* are derived from the same background to lack expression of E1A and E1A Δ 4-25, respectively. HAdV-5 *d/520* and *d/1131/520* express only the 12S product of E1A, with the latter containing an additional C-terminal deletion (aa 219 to 289) (52). WT serotypes of HAdV-3 (strain GB, lot 11W), -4 (strain RI-67, lot 3W), -9 (strain Hicks, lot 1W), and -12 (strain Huie, lot 8W) were purchased from the ATCC via Cedarlane. A549 cells were infected at a multiplicity of infection (MOI) of 5 PFU/ml (for HAdV-5) or 25 PFU/ml for all other virus species to attain similar levels of infection. Cell cultures were infected at ~50% confluence, and subconfluent cells were collected at the indicated time points for downstream experiments. For plaque assays, HEK293 cells were infected with serially diluted samples for 1 h at 37°C before being overlaid with DMEM containing 1% SeaPlaque agarose (Lonza).

RNAi knockdown. Downregulation of PKA subunits R1 α , R11 α , and $C\alpha$ was performed using Silencer select siRNA (Thermo Fisher Scientific), specifically s286 (PRKAR1A), s11086 (PRKAR2A), and s11065 (PRKACA). Four hours after seeding, siRNA was delivered to A549 and HT1080 cells via transfection with Silentfect (Bio-Rad) according to the manufacturer's instructions. A scrambled siRNA was used as a negative control. Treated cells were used for experiments 48 h posttransfection.

Plasmids. All constructs were expressed in vectors under the control of the cytomegalovirus (CMV) promoter, with the exception of the luciferase reporter, which was driven by the cAMP-responsive rat PEPCK promoter. WT R1 α , R11 α , and $C\alpha$, along with Δ D/D mutants, were previously described (9). WT full-length HAdV-52 E1A was synthesized using IDT's gene synthesis platform from a published genome sequence (20). E1A fragments, truncations, and deletion mutants for all species were expressed as fusions

to EGFP and either previously described (9) or derived via PCR and cloned into pEGFP-C2. Chimeras (HAdV-4 chimera and HAdV-5 chimera) and point mutants (HAdV-5 D21E, E26T, V27L and HAdV-3 E22D, T27E, L28V) were cloned similarly.

Western blotting and coimmunoprecipitation. Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 0.1% NP-40) with protease inhibitor cocktail. Protein concentrations were determined using Bio-Rad protein assay reagent using bovine serum albumin (BSA) as a standard. Coimmunoprecipitations were carried out at 4°C for 4 h. Two percent of the sample was kept as input control. After washing with NP-40 buffer, complexes were boiled in 25 μ l of LDS sample buffer for 5 min. Samples were separated on NuPage Bis-Tris gels (Life Technologies) and transferred onto a polyvinylidene difluoride membrane (Amersham). Membranes were blocked in 5% skim milk or 3% BSA constituted in Tris-buffered saline (TBS) with 0.1% Tween 20. Primary antibodies used include mouse α -RI α (BD), mouse α -RII α (BD), mouse α -C α (BD), rabbit α -actin (Sigma), rabbit α -EGFP (Clontech), rat anti-hemagglutinin (α -HA; clone 3F10; Roche), mouse α -MYC (clone 9E10; in-house), rabbit α -CREB1 (Santa Cruz), rabbit α -CREB1 p-Ser133 (Santa Cruz), and mouse α -E1A (clone M73; in-house). Horseradish peroxidase-conjugated secondary antibody was detected using Luminata Forte or Crescendo substrate (Millipore).

Immunofluorescence microscopy. Cells were fixed in 3.7% paraformaldehyde, permeabilized on ice using 0.2% Triton X-100, and blocked using 3% BSA in phosphate-buffered saline (PBS). Samples were incubated in the primary antibody (rabbit α -RI α [Thermo], rabbit α -RII α [Santa Cruz], rabbit α -C α [Santa Cruz], rabbit α -cAMP [Abcam], rabbit α -CREB1 p-Ser133 [Santa Cruz], mouse α -DBP [clone B6-8; in-house], and mouse α -E1A [clone M73 or M37; in-house]) for 1 h at room temperature or 4°C overnight and another hour at room temperature with secondary antibody (Alexa Fluor 594 or 488; Life Technologies). Samples were mounted with Prolong Gold reagent containing 4',6-diamidino-2-phenylindole (DAPI; Life Technologies). Confocal images were acquired using a Fluoview 1000 laser scanning confocal microscope (Olympus Corp). Quantification of total cellular signal and nuclear signal was conducted using ImageJ. Cells were normalized for both cytoplasmic and nuclear size, and percent nuclear signal was determined as previously described (53).

Virus replication assay. A549 cells were treated with siRNAs, followed by HAdV infection for 1 h at 37°C at the indicated MOI. Total cell DNA was purified at 6 and 48 h postinfection using a DNeasy blood and tissue kit (Qiagen). Viral DNA levels were quantified by qPCR with Power SYBR green (Thermo) using a forward (F) primer (E1A-F, AGAGGCCACTCTTGAGTGC) that recognizes a conserved sequence in E1A in combination with a serotype-specific reverse (R) primer (Ad3E1A-R, TACAGATCGTGCAGCGTAGG; Ad4E1A-R, AGCGAAGGTGTCTCAATGG; Ad5E1A-R, CGTCACGTCTAAATCATAC; Ad9E1A-R, GGGCATCTACCTCCAGATCA; Ad12E1A-R, CGGCAGACTCCACATCAAG). Values were normalized to GAPDH (F, ACTGCTT AGCACCCTGGCCAA; R, ATGGCATGGACTGTGGTCATGAGTC), and the fold increase of viral copy number at 48 h was calculated by normalizing to input viral DNA at 6 h postinfection. Viral replication efficiency in the presence of PKA knockdowns was presented as the relative value compared to those for scrambled control siRNA-treated cells, which were normalized to 1.

Luciferase reporter assay. HT1080 cells were cotransfected with a constitutive β -galactosidase reporter plasmid for normalization, a plasmid containing the luciferase gene driven by the rat PEPCK promoter, and the indicated EGFP-E1A fusion constructs. The reporter was validated by 6 h of 0.5 μ M forskolin (fsk) treatment and siRNA-mediated knockdown of PKA protein. Twenty-four hours posttransfection, cells were lysed in 200 μ l of the supplied lysis buffer (Promega). For detection of luciferase production, 50 μ l of lysate was mixed with 50 μ l of the provided luciferase substrate (Promega) immediately before detection of light was measured using a Berthold Lumat LB 9507. Relative light values were set to an empty plasmid control, were normalized to protein levels via Bradford assay, and were further normalized via β -galactosidase activity as detected by ONPG (ortho-nitrophenyl- β -D-galactopyranoside).

PKA activity assay. PKA activity in cell lysates was measured using the protein kinase A colorimetric activity kit (Thermo). A total of 1×10^6 A549 cells were seeded overnight and then either treated with fsk for 6 h or infected with the indicated virus. After harvesting at the indicated time points, PKA activity of the cell lysates was assayed using the protocol outlined by the manufacturer. Activity in uninfected cells was normalized to 1 and compared to all other conditions.

Statistical analysis. All experiments were carried out with three biological replicates performed in duplicate. Graphs represent means and standard errors of the means (SEM) for all biological replicates. For Western blotting, a representative image was selected. Statistical significance of numerical differences was calculated using *t* tests (Fig. 11) or one-way analysis of variance and Holm-Sidak *post hoc* comparisons (Fig. 4, 12, and 13) between experimental conditions.

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