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Brief Communication

Functional analysis of the C-terminal region of human adenovirus E1A reveals a misidentified nuclear localization signal

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ABSTRACT

The immortalizing function of the human adenovirus 5 E1A oncoprotein requires efficient localization to the nucleus. In 1987, a consensus monopartite nuclear localization sequence (NLS) was identified at the C-terminus of E1A. Since that time, various experiments have suggested that other regions of E1A influence nuclear import. In addition, a novel bipartite NLS was recently predicted at the C-terminal region of E1A *in silico*. In this study, we used immunofluorescence microscopy and co-immunoprecipitation analysis with importin-α to verify that full nuclear localization of E1A requires the well characterized NLS spanning residues 285–289, as well as a second basic patch situated between residues 258 and 263 (^{258}RVGGRRQAVECIEDLLNEPGQPLDLSCKRPRP^{289}). Thus, the originally described NLS located at the C-terminus of E1A is actually a bipartite signal, which had been misidentified in the existing literature as a monopartite signal, altering our understanding of one of the oldest documented NLSs.

Introduction

The human adenovirus (HAdV) E1A oncoprotein is the first viral protein to be expressed following infection (Pelka et al., 2008). E1A plays a critical role in reprogramming the infected cell by modulating host transcriptional machinery to force quiescent cells to enter the cell cycle and suppress the cellular innate antiviral responses thereby providing an optimal environment for viral replication (Bayley and Mymryk, 1994; Flint and Shenk, 1997). E1A has no enzymatic or specific DNA binding capabilities and instead carries out its functions by binding to and manipulating a plethora of key cellular regulatory proteins through short linear motifs found within its 289 amino acid sequence (Avvakumov et al., 2002; Pelka et al., 2008). Based on amino acid similarity among different HAdV species, there are four regions of high conservation within the E1A sequence termed conserved regions 1–4 (CR1–CR4) and it is typically within these regions that the linear motifs are located (Pelka et al., 2008; Avvakumov et al., 2004). This strategy allows E1A to interact with over 50 cellular targets and associate with over 17,000 genomic promoters (Ferrari et al., 2008, 2009.

The specific subcellular localization of any protein is essential for its given functions. Proteins targeted to the nucleus contain nuclear localization signals (NLSs) that typically interact in the cytosol with the importin-α family of NLS receptors (also known as karyopherin α) (Macara, 2001). Importin α recognizes two classes of NLSs: monopartite NLSs, which have a single cluster of basic amino acid residues and bipartite NLSs, having two clusters of basic residues separated by a linker region of 10–25 amino acids (Lange et al., 2007, 2010). The prototypical monopartite signal is exemplified by the SV40 Large T antigen (T-Ag) NLS (^{126}PKKKRKV^{132}) (Kalderon et al., 1984), while bipartite signals are exemplified by the *Xenopus laevis* nucleoplasmin NLS (^{155}KRPAATKKAGQAKKKK^{170}) (Dingwall et al., 1988).

Translocation from the cytoplasm to the nucleus is a necessary process for E1A to gain access to the members of its target complexes (Madison et al., 2002; Douglas and Quinlan, 1995). E1A contains a highly conserved monopartite NLS, a conserved five amino acid sequence mapped to the extreme C-terminus of E1A (^{285}KRPRP^{289}) (Fig. 1) that preferentially interacts with importin alpha 3 (Qip1) (Lyons et al., 1987; Kohler et al., 2001). A second non-canonical NLS was identified in CR3 of HAdV-5 E1A with the consensus sequence FV(X)7-26MXSLYXYM(X)$_4$MF (Standiford and Richter, 1992; Slavicek et al., 1989). Unlike the C-terminal NLS, this sequence is not conserved and is unique to HAdV-5 E1A. However, using a genetic assay in *Saccharomyces cerevisiae*, we recently showed that the CR3 region from all HAdV species is able to induce

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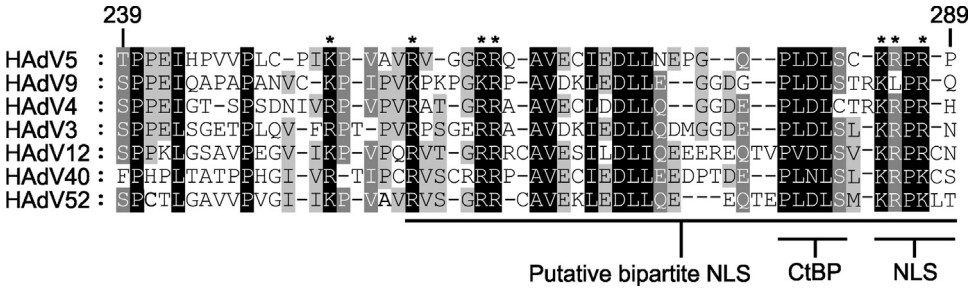

Fig. 1. Amino acid sequence alignment of CR4 across HAdV species. An amino acid sequence alignment of E1A CR4 from seven HAdV types representing the seven HAdV species (A–G) reveals a second cluster of basic residues upstream of the canonical C-terminal NLS starting at K253. Basic residues are indicated with *. Darker shading corresponds to higher sequence homology. The previously identified monopartite NLS is indicated, as the CtBP binding motif (PLDLS) and the putative bipartitite NLS.

nuclear localization, indicating the presence of a non-canonical NLS which does not follow the previously reported consensus sequence. In this same study we reported yet another novel non-canonical NLS in the N-terminal region of E1A mapped to residues 30–69 (Marshall et al., 2014).

Previous analysis has shown that efficient nuclear localization of E1A is required for the immortalization and transformation by E1A in cooperation with HAdV E1B (Douglas and Quinlan 1994, 1995). These studies also revealed that inexplicably, mutations in other regions within the second exon of E1A distal from the canonical C-terminal NLS were deficient for both nuclear localization and transforming ability with E1B. We recently conducted an extensive mutational analysis of the C-terminal region of E1A as a means of identifying key amino acid residues required for interaction with several cellular targets. During this study, we observed that the amino acid substitution at the highly conserved residues 262 and 263 (R262/263E) of HAdV E1A resulted in a deficiency in nuclear localization (Cohen et al., 2013). The localization phenotype of this double point mutant was consistent with that of the previously described E1A mutant with a deletion that spans residues 256–273 (Douglas and Quinlan, 1994). Furthermore, the R262/R263E mutant was unable to interact with Qip1 in co-immunoprecipitation (co-IP) assays. Other highly conserved basic residues are also situated within this region of E1A (K253 and R258 specifically) (Fig. 1) and interestingly, a bipartite NLS was predicted for E1A *in silico*, spanning residues 258–289 (Cohen et al., 2013).

In the current study, we have verified that the C-terminal region of E1A indeed contains a bona fide bi-partite NLS (^{258}RVGGRRQAVECIEDLLNEPGQPLDLSCKRPRP^{289}) that is both necessary and sufficient for nuclear localization.

Results

The C-terminal E1A NLS requires both the major and minor binding groove of Qip1

Importin-α contains two NLS binding grooves, a major site located at the N-terminal Armadillo (Arm) repeat 2–4, and a minor site located at the Arm repeat 7–8. Classical monopartite NLSs specifically bind to the major binding site of Qip1, whereas bipartite NLSs bind to both sites (Conti et al., 1998; Dingwall and Laskey, 1998). To determine which binding sites the C-terminal E1A NLS interacts with, we used Qip1 variants lacking the importin-β binding (IBB) domain, which is an autoinhibitory region, and point mutants that specifically disrupt the major (ΔMajor) or minor (ΔMinor) NLS binding sites, respectively (Conti et al., 1998; Dingwall and Laskey, 1998; Lange et al., 2007). Human HT1080 fibrosarcoma cells were transfected with vectors co-expressing GFP fusions of the monopartite NLS from the SV40 T-Ag,

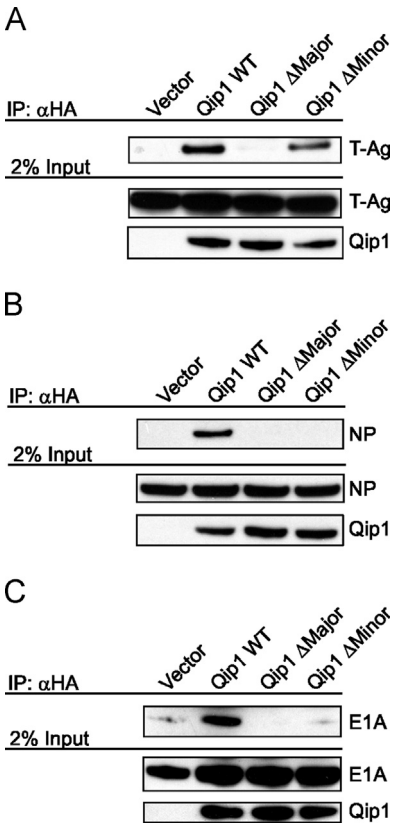

Fig. 2. The C-terminus of E1A binds importin-α like a bipartite NLS. Human HT1080 cells were co-transfected with vectors expressing GFP-fused to: A) the monopartite NLS from SV40 Large-T antigen (T-Ag; PKKKRKV), B) the bipartite nucleoplasmin NLS (KRPAATKKAGQAKKKK), or C) the C-terminal region of E1A (residues 187–289) along with the indicated HA-tagged Qip1 variants. Lysates were immunoprecipitated using anti-HA antibodies (12CA5) and immunoblotted using anti-GFP antibodies.

the bipartite NLS from the nucleoplasmin protein (NP), or the second exon encoded portion of E1A (residues 187–289) and the panel of HA-tagged Qip1 constructs listed above. Lysates were immunoprecipitated using anti-HA antibodies (clone 12CA5) and subsequently immunoblotted for each GFP fusion (Fig. 2). As expected, the monopartite T-Ag NLS failed to interact with Qip1 ΔMajor (Fig. 2A), while retained binding to Qip1 ΔMinor. The bipartite nucleoplasmin NLS was unable to interact with either Qip1 ΔMajor or ΔMinor, as anticipated (Fig. 2B). Like the bipartite nucleoplasmin NLS, the C-terminal E1A NLS failed to bind to either Qip1 mutant (Fig. 2C). These results indicate that the C-terminal E1A NLS requires both the major and minor NLS binding sites of Qip1 to maintain its interaction, which is indicative of the presence of a bipartite NLS.