Notch1 augments intracellular trafficking of adeno-associated virus 2

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ABSTRACT

We report here the significance of Notch1 receptor in intracellular trafficking of recombinant adeno-associated virus 2 (rAAV). RNA profiling of human prostate cancer cell lines with varying degrees to AAV transduction indicated a correlation of the amount of Notch1 to rAAV transgene expression. A definitive role of Notch1 in enhancing AAV transduction was confirmed by developing clonal derivatives of DU145 cells overexpressing either the full-length or the intracellular Notch1. To discern stages of AAV2 transduction influenced by Notch1, competitive binding with soluble heparin, Notch1 antibody, intracellular trafficking using Cy3-labeled rAAV2, and blocking assays for proteasome and dynamin pathways were performed. Results indicated that in the absence or low-level expression of Notch1, only binding of virus was found on the cell surface but internalization was impaired. However, increased Notch1 expression in these cells allowed efficient perinuclear accumulation of labeled capsids. Nuclear transport of the vector was evident by transgene expression and real-time PCR analyses. Dynamin level was not found to be different among these cell lines but blocking dynamin function abrogated AAV2 transduction in DU145 clones overexpressing the full-length Notch1 but not in clones overexpressing the intracellular Notch1. These studies provide evidence for the role of activated Notch1 in intracellular trafficking of AAV2, which may have implications in the optimal use of AAV2 in human gene therapy.
Recombinant adeno-associated virus (rAAV) has attracted considerable interest as an efficient and safe gene therapy vector. The most commonly studied AAV vector for gene transfer is derived from serotype 2 (5,8,16). Initial binding and internalization of AAV2 has been attributed to heparan sulfate proteoglycan receptor (21), and αvβ5 integrin and fibroblast growth factor receptor-1 coreceptors (14,22). Once internalized, nuclear trafficking and transgene expression following complementary strand formation are known to be aided by many cellular factors such as dynamin, Rac1, phosphatidylinositol 3-kinase (PI3-K), and T cell protein tyrosine phosphatase (9,13,17,18).

The present study demonstrates that Notch1 plays an important role in mediating intracellular trafficking and nuclear transport of AAV2. Notch-1 is a transmembrane receptor, expressed as heterodimeric protein after intracellular processing of the full-length protein (1). Notch protein requires three cleavage steps to become fully functional (4). Engagement by ligand results in the cleavage of Notch heterodimer, releasing the intracellular domain of Notch and allowing translocation to the nucleus. Notch mediated cell-cell interaction and signaling are important for stem cell maintenance, cell fate determination, cell proliferation and differentiation in a variety of tissues. The significance of Notch pathway in proliferation and gene expression of Epstein-Barr virus, Kaposi’s sarcoma-associated herpesvirus, adenovirus, human papilloma virus, and SV40 has been previously reported (6,7,10-12,15). In addition to its role in promoting gene expression, activated Notch has been shown to effect nuclear trafficking of ubiquitin ligase protein and activate PI3-K, Rho and Rac proteins (2,20). Previous studies have demonstrated that endosomal escape and nuclear localization of AAV2 is mediated by these proteins and a role for dynamin in the release of endosomally encapsulated vector (3,9). Further, activation of full-length Notch1 is also known to involve dynamin interaction (19).
Gene expression profile correlating to rAAV2 transduction. In our studies on AAV2 transduction to human epithelial prostate cancer cells, we observed a wide variation in transduction efficiency. Whereas PCa2b cells indicated highest AAV2 gene transfer, moderate amount of rAAV2 transgene expression was seen in LNCaP cells and much lower expression in DU145 cells (Fig 1A). As a first step towards identifying specifically active genes in these cells, RNA microarray analysis was performed using the Affymetrix gene chip from 293, PCa2b, DU145 and M07e cells. Clustering of the data, correlating to reporter gene expression, resulted in the identification of 6 differently expressed genes. Out of these, one encoded a structural protein (desmoplakin [DSP]) and 5 proteins with functional significance (SRY box 9 [SOX9]; n-myc downstream-regulated gene1 [NDRG1]; chloride channel protein 3 [CLCN3]; phosphatidic acid phosphatase type 2A [PPAP2A]; and Notch1) at the membrane, cytoplasmic or nuclear compartments.

Notch1 expression correlates to rAAV2 transduction. To determine if any of the above proteins influences rAAV2 transduction, we carried out either blocking studies with antibodies (for CLCN3, PPAP2A and DSP proteins) or siRNA (for Notch1) in cells which showed upregulation, or overexpression using expression vectors (for SOX9 and NDRG1) in cells which showed low-level expression. Data from these studies indicated significant influence on AAV2 transduction only following siRNA inhibition of Notch1 as shown in Fig. 1B. rAAV2 transduction in Notch siRNA transfected PCa2b and 293 cells was inhibited by 75.6% (p<0.0007) and 97.5% (p<0.0001) respectively. Altering the expression of other five proteins did not significantly change the efficiency of rAAV2 transduction (data not shown). This data strongly suggested a correlation of the amount of Notch1 transcript to rAAV2 transduction.
Establishment of stable cell lines expressing the full-length or the intracellular domain of Notch1 and characterization of rAAV2 transduction. In the next set of experiments, we sought to determine if overexpression of Notch1 in DU145 cells would render them more permissive for rAAV2 transduction. To determine whether the full-length or the intracellular Notch1 is responsible for enhancing rAAV2 transduction, we developed independent clones of DU145 expressing either the full-length (DU145-hN1) or the intracellular Notch1 (DU145-hN1IC) by G418 selection and tested for augmenting rAAV2 transduction by infecting with 100 MOI of rAAV-2 encoding luciferase. Results showed a significant increase in luciferase expression in DU145 clones overexpressing either the full-length or the intracellular domain of Notch1 (p<0.02; Fig. 2A). Quantitative real-time PCR for vector genome indicated significantly higher copy number of rAAV2 DNA in DU145-hN1 and DU145-hN1IC cells compared to the parental DU145 cells following transduction with the same vector MOI (Fig. 2B). Competitive binding with Notch1 antibody did not block vector transduction indicating Notch1 does not serve as a receptor or co-receptor for virus binding.

Functional characterization of Notch1 with fluorescently labeled virus. In the next step, fluorescently labeled rAAV-2 GFP was produced to study viral intracellular trafficking and was used to infect replicate cultures of HeLa, DU145 and DU145-hN1 and DU145-hN1IC clones either at 4°C or at 37°C. The cells were fixed at different time points (0.5, 1, and 2 hrs) and analyzed by confocal microscopy. Results of these studies are provided in Fig. 3A. In all cell types, Cy3-labeled virus was found to bind to the cell surface at 4°C. When incubation was continued at 37°C, internalization of the virus and nuclear trafficking was seen in HeLa, DU145-hN1 and DU145-hN1IC cells. There was also higher amount of internalized vector in DU145-hN1IC cells compared to DU145-hN1 cells. However, there was very minimal vector
internalization in unmodified DU145 cells. After 0.5 hrs of incubation at 37°C, the majority of internalized virus in permissive cells was found in the cytoplasm and a few virus particles were localized in the nuclei. Almost half of the virus particles was localized in the nuclei after 1 hr incubation at 37°C and by 2 hrs at 37°C, most of the virus particles were accumulated in the nuclear area as detected by counterstaining with Hoechst 33258 (Fig. 3B). However, such vector entry or nuclear trafficking was not observed in the parental DU145 cells even after 2 hrs of incubation at 37°C. Inhibition of proteasomal degradation did not augment Notch1 enhancement of rAAV-2 transduction (data not shown).

**Role of dynamin in Notch1 regulated rAAV2 transduction enhancement.** Proteolytic cleavage of full-length Notch1 to active, intracellular form is mediated in part by dynamin. To establish whether low abundance of Notch1, correlating to lower rAAV2 transduction, is due to insufficient expression of dynamin, the amount of dynamin in HeLa, DU145, DU145hN1, DU145hN1IC, and 293 cells were determined by Western blot analysis. Results, shown in Fig. 4A, indicated comparable amounts of dynamin expression between all cell types and that low amount of Notch in DU145 cells was not because of the absence of dynamin. To further determine whether the influence of Notch1 in enhancing rAAV2 transduction was dependent on dynamin function, a dominant negative mutant dynamin was overexpressed using a recombinant adenovirus vector (9). As shown in Fig. 4B, it was interesting to note that blocking dynamin function greatly inhibited rAAV2 transduction only in DU145hN1 clone but not in DU145-hN1IC clone confirming that the proteolytically cleaved intracellular form of Notch1 is responsible for the observed rAAV transduction enhancement.
Collectively, these studies provide evidence that Notch1 plays a significant role in nuclear trafficking of rAAV2 and modulation of Notch expression may give additional options for increasing AAV2 gene transfer efficiency in target cells with limitations in AAV trafficking and/or nuclear transport of the vector.
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REFERENCES


FIGURE LEGENDS

Figure 1. Variation in rAAV2 transduction efficiency in human epithelial prostate cancer cells and inhibition of rAAV2 transduction by Notch1 siRNA. (A) 293, PC2b, DU145 and M07e cells were transduced with 1 MOI of rAAV2-GFP. Transgene expression was monitored in a fluorescent microscope 48 hrs later. (B) Monolayer cultures of 293 and PC2b were transfected with synthetic siRNA oligonucleotide for human Notch1 following which the cells were infected with 100 MOI of rAAV2-luciferase. Forty eight hours later, the cells were lysed and luciferase activity measured. Data shown indicated percentage inhibition in luciferase activity in Notch1 siRNA transfected cells (■) compared to cells with no siRNA (□). The assay was done in triplicate.

Figure 2. rAAV transduction in DU145 cell overexpressing either the full-length or intracellular domain of Notch1. (A) DU145 and DU145 clones overexpressing either the full-length or the intracellular Notch1 were transduced with 100 MOI rAAV2-luciferase in triplicate. The cells were harvested 48 hrs after transduction and luciferase activity determined. Relative light units (RLU) as a measure of luciferase activity was normalized to protein content in each lysate (*p<0.02 when compared to luciferase activity in DU145 cells). (B) Semi-quantitative real-time PCR was performed with DNA isolated from DU145 cells or DU145 cells overexpressing the full-length or intracellular Notch1. Values shown represent fold increase in vector copy number per cell from each clone compared to that from DU145 cells.

Figure 3. Transduction of Cy3-labeled rAAV2-GFP in DU145 cells overexpressing Notch1. (A) Time course analysis of Cy3-labeled AAV2 intracellular trafficking. DU145, DU145-hN1, DU145-hN1IC, and HeLa cells were incubated with Cy3-labeled rAAV at 4°C for binding.
The cells were either washed and fixed after 1 hr or shifted to 37°C for 0.5, 1, and 2 hrs prior to fixation. Tracking of virus binding and intracellular trafficking were analyzed by confocal microscopy. (B) **Nuclear accumulation of Cy3-labeled rAAV-2.** Cells were counterstained with Hoechst 33258 after 2 hrs of incubation at 37°C to demonstrate nuclear accumulation of the viral particles.

**Figure 4. Dynamin expression in different cell lines and rAAV2 transduction following blocking of dynamin function.** (A) Equal amounts of protein from lysates from indicated cells were separated by SDS-PAGE and detected using antibody for human dynamin. The same blot was rehybridized with β-actin antibody to confirm quantity of protein in each lane. (B) Dynamin function in DU145hN1 and DU145hN1IC cells was blocked by transducing rAd encoding a dominant-negative mutant dynamin following which they were transduced with rAAV2-luciferase. Luciferase activity was determined 48 hrs later from cell lysates and normalized to protein concentration. Data from each cell line is represented as a reduction in the percentage of luciferase activity compared to rAAV2-luciferase transduction without dynamin blocking.
Figure 1A
Figure 1B

Percent Luciferase Expression

293  PCa2b

$p<0.0001$  $p<0.0007$
Figure 2A
Figure 2B

- Fold increase/cell
- DU145-hN1
- DU145-hN1IC
Figure 3A
Figure 3B
Figure 4A

- Dynamin
- β-actin
Figure 4B