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Gene transfer with adeno-associated virus 2 vectors: the growth factor receptor connection

Review Article

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Summary

Adeno-associated virus 2 (AAV)-based vectors have gained attention as a potentially useful alternative to the more commonly used retroviral and adenoviral vectors for human gene therapy. However, there are at least two major obstacles that limit high-efficiency transduction by AAV vectors. The first relates to the extent of expression of the cellular receptor for AAV, and the second concerns the rate-limiting step of the viral second-strand DNA synthesis. With reference to the first obstacle, although the ubiquitously expressed cell surface heparan sulfate proteoglycan (HSPG) has been reported to be a receptor AAV, HSPG alone is insufficient for AAV infection, and human fibroblast growth factor receptor 1 (FGFR1) has been identified as a co-receptor for successful viral entry into the host cell. With reference to the second obstacle, a cellular protein, designated the single-stranded D-sequence–binding protein (ssD-BP), phosphorylated at tyrosine residues, has been identified which plays a crucial role in viral second-strand DNA synthesis. The ssD-BP is phosphorylated by the protein tyrosine kinase activity of the human epidermal growth factor receptor (EGFR). Thus, both FGFR1 and EGFR are crucial determinants in the life cycle of AAV, and further studies on the interaction between the FGFR and EGFR may yield new insights not only into its role in the host cell but also in the optimal use of AAV vectors in human gene therapy.

I. Introduction

The non-pathogenic nature of the adeno-associated virus 2 (AAV), a single-stranded DNA-containing human parvovirus (Srivastava et al., 1983), coupled with the remarkable site-specific integration of the wild-type (wt) AAV genome into the human chromosome 19 (Kotin et al., 1990, Samulski et al., 1991), generated a significant interest in the development of AAV vectors as a potentially useful alternative to the more commonly used retrovirus and adenovirus vectors in human gene therapy (Berns and Giraud, 1996). Indeed, AAV vectors have been successfully used for gene transfer *in vitro* as well as *in vivo*, and are currently in Phase II clinical trials for gene therapy of cystic fibrosis (Flotte and Carter, 1997). Although AAV possesses a broad host-range that transcends the species barrier (Muzyczka, 1992), the efficiency of AAV-mediated transduction has been reported to vary widely. Recently, the ubiquitously expressed cell surface heparan sulfate proteoglycan (HSPG) was identified as a receptor for AAV (Summerford and Samulski, 1998), it has become increasingly clear that HSPG alone is insufficient for AAV infection. For example, our recent studies have documented a significant donor variation in terms of the ability of AAV vectors to transduce primary human bone marrow-derived $CD34^+$ hematopoietic progenitor cells (Ponnazhagan et al., 1997). In these

studies, AAV-mediated transgene expression ranged between 15-80% of infected cells from approximately 50% of normal volunteer donors, whereas AAV failed to bind to $CD34^+$ cells from approximately 50% of donors and consequently, cells from these donors could not be transduced. Similarly, we have reported that the efficiency of AAV transduction in permissive cells does not correlate with the receptor number, and that a cellular protein, designated as the single-stranded D-sequence-binding protein (ssD-BP), phosphorylated at tyrosine residues, plays a crucial role in the viral second-strand DNA synthesis (Qing *et al.*, 1997; 1998), a rate-limiting step in AAV-mediated transgene expression (Fisher et al., 1996; Ferrari et al., 1996). Thus, the two obstacles encountered in attempting to obtain high-efficiency transduction by recombinant AAV vectors will be discussed briefly as follows.

II. The first obstacle

A. Successful infection of cells by AAV requires fibroblast growth factor receptor 1 (FGFR1) as a cell surface co-receptor

It has previously been demonstrated that all cell types which bind AAV can also be infected by AAV (Ponnazhagan et al., 1996, 1997; Qing et al., 1998; Summerford and Samulski. 1998; Bartlett and Samulski, 1998). For example, human cell lines such as HeLa, KB, and 293, which have been shown to be permissive for AAV infection, can bind AAV, whereas non-permissive cells, such as M07e, cannot. Interestingly, however, we noted that murine NIH3T3 cells, which could not be transduced by a recombinant AAV vector, could bind AAV quite efficiently. As NIH3T3 cells are known to express HSPG (Ledoux et al., 1992), this observation suggested that in addition to HSPG as a primary receptor for binding, AAV might require a putative cell surface co-receptor for efficient entry. Since fibroblast growth factor (FGF) has an absolute requirement for HSPG prior to efficient binding to the fibroblast growth factor receptor (FGFR) (Green et al., 1996), we reasoned that FGFR might be a potential candidate. We examined human cell types known to be non-permissive for AAV infection, such as the human megakaryocytic cell line M07e (Ponnazhagan et al., 1996), as well as those that do not express either HSPG or FGFR, such as the human lymphoblastoid cell line Raji (Kiefer et al., 1990; Lebakken and Rapraeger, 1996). These cell types were stably transfected with cDNA expression plasmids containing either the murine HSPG core protein (Syndecan-1) (Saunders et al., 1989), or the human FGFR1 (Johnson et al., 1990), or both, followed by the determination of radiolabeled FGF-binding, radiolabeled-AAV binding, and recombinant AAV-mediated transgene expression. M07e cells, known to lack HSPG expression (Bartlett and Samulski, 1998), could not bind FGF. Stable transfection with huFGFR1 cDNA alone allowed for a low-level of FGF binding, the extent of which was significantly higher when M07e were co-transfected with both HSPG and FGFR1 cDNAs. Interestingly, transfection with the muHSPG cDNA alone resulted in significant binding of FGF. These results suggest that M07e cells do indeed express the endogenous FGFR gene. As expected, mock-transfected Raji cells also failed to bind FGF as they lack both HSPG and FGFR. Only low levels of FGF binding were detected in Raji clones stably transfected with either the HSPG or FGFR1 expression plasmids alone, whereas in Raji cells co-expressing both, a significant binding of FGF occurred, further corroborating the requirement of both HSPG and FGFR1 for ligand binding. It was also interesting to note that the binding patterns of AAV to both the M07e and Raji cells co-expressing HSPG+FGFR1 genes closely resembled that of FGF binding. Taken together, these results strongly suggested that cell surface expression of both HSPG and FGFR1 is required for successful binding of AAV to the host cell (Qing et al., 1999).

In order to determine whether non-permissive cells could be rendered positive for AAV transduction following stable transfection with cDNAs encoding muHSPG, or huFGFR1, or both, individual clonal isolates from both cell types were either mock-infected or infected with a recombinant AAV vector under identical conditions and analyzed for transgene expression by fluorescence-activated cell-sorting (FACS). Whereas little transgene expression was seen in mock-infected M07e cells, as expected, it was evident that Mo7e cells expressing either HSPG alone, or both HSPG and FGFR1, but not FGFR1 alone, could be readily transduced by the recombinant AAV vector. Expression of the exogenous HSPG in M07e cells was sufficient to render the cells permissive to AAV infection because M07e cells express the endogenous FGFR gene. On the other hand, Raji cells failed to be transduced by recombinant AAV if only the exogenous HSPG or FGFR1 genes were expressed, but co-expression of both HSPG and FGFR1 conferred AAV infectivity to these cells, albeit at a relatively low-efficiency. Inclusion of additional individual clonal isolates from both cell types yielded very similar results. These studies establish that co-expression of both HSPG and FGFR1 is required both for binding and also entry of AAV into the host cell (Qing et al., 1999).

B. FGFR autophosphorylation is not required for AAV-mediated transduction

Since ligand binding to FGFR consequently leads to receptor dimerization followed by ion activation of the FGFR-associated protein tyrosine kinase (PTK),

ultimately resulting in recruitment of intracellular signaling molecules (Rapraeger et al., 1991; Ledoux et al., 1992; Roghani and Moscatelli; 1992, Givol and Yayon, 1992; Kan et al., 1993), it was of interest to investigate whether FGFR PTK activity affected AAV-mediated transgene expression. To this end, cells permissive for AAV infection, human 293 and HeLa cells, were either mock-treated, or first treated with specific inhibitors of FGFR PTK (Mohammadi et al., 1997) followed by infection with a recombinant AAV vector under identical conditions and the extent of transgene expression was determined as described above. These experiments demonstrated that none of the FGFR PTK inhibitors tested had any significant effect on AAV-mediated transgene expression. From these studies, we conclude that FGFR PTK activity is not required for AAV-mediated transgene expression (Qing et al., 1999).

C. FGF treatment perturbs AAV binding to non-permissive as well as permissive cells, and abrogates viral entry into permissive cells

The following experiments further supported the contention that FGFR1 acts as a co-receptor for AAV biding and entry. First, we hypothesized that treatment of non-permissive cells such as NIH3T3 cells, and permissive cells such as 293 cells, with large excess of FGF would perturb the ability of AAV to bind to the host cell. Binding studies with NIH3T3 and 293 cells were carried out using radiolabeled AAV in the presence or absence of excess amounts of FGF, with additional controls including wt AAV or heparin (as positive controls) and EGF (as a negative control). The results of these experiments documented that AAV binding to NIH3T3 cells was inhibited by heparin, as expected (Summerford and Samulski, 1998), and FGF also inhibited AAV binding to a significant extent, whereas EGF had no effect under identical conditions. As expected, unlabeled wt AAV significantly inhibited binding of radiolabeled AAV to 293 cells. Likewise, excess FGF was also able to reduce AAV binding to 293 cells. On the other hand, as with the NIH3T3 cells, similar concentrations of EGF had no significant effect on AAV binding to 293 cells. Second, we reasoned that excess amounts of FGF might perturb AAV infection. To this end, equivalent numbers of 293 cells were infected with a recombinant AAV vector either in the absence or presence of excess FGF or EGF, under identical conditions. Forty-eight hrs post-infection, transgene expression was evaluated by X-gal staining as previously described (Ponnazhagan et al., 1996; 1997). The results of these experiments indicated that AAV-mediated transduction of 293 cells was inhibited in the presence of FGF by approximately 89%, but in the presence of EGF by only 2%. The lack of transgene expression in the presence of FGF was not due to phosphorylation of the ssD-BP in 293 cells as these assays carried out with prior treatment with genistein also resulted in similar results (89% inhibition with FGF, 0% inhibition with EGF). Taken together, these results strongly suggest that HSPG-FGFR1 interaction is crucial not only for binding, but also for entry of AAV into the host cell. Based on all available information, we propose a model for the initial step in AAV infection, which is depicted in **Figure 1**. In this model, co-expression of cell surface HSPG and FGFR1 is required for successful AAV binding followed by viral entry (Panel A), both of which are blocked by FGF (Panel B) (Qing et al., 1999).

III. The second obstacle

A. Inhibitors of epidermal growth factor receptor (EGFR) protein tyrosine kinase (PTK) activity increase the transduction efficiency of recombinant AAV

We have previously shown that inhibition of tyrosine phosphorylation of the ssD-BP by genistein, a specific inhibitor of all protein tyrosine kinases (Akiyama et al., 1987; Barnes and Peterson, 1995; Constantinou and Huberman, 1995; Carlo-Stella et al., 1996), increased transduction efficiency by recombinant AAV (Qing et al., 1997). To investigate which kinase may be responsible for tyrosine phosphorylation of the ssD-BP, we studied the effects of various kinase inhibitors such as apigenein (MAP kinase) (Kuo and Yang, 1995), herbimycin A ($pp60^{c\text{-}src}$) (Fukazawa et al., 1991), LY294002 (PI 3-kinase) (Vlahos et al., 1994), staurosporine (CaM kinase, MLC kinase, PK-A, PK-C, PK-G) (Couldwell et al., 1994), tyrphostin A48 (EGF-R PTK) (Gazit et al., 1989), wortmannin (MAP kinase, MLC kinase, PI 3-kinase, PI 4-kinase) (Okada et al., 1994), in addition to genistein, on the transduction efficiency of recombinant AAV. Following treatment with these reagents, cells were infected with a recombinant AAV vector, followed by staining with X-gal 48 hrs post-infection. The results indicated that in addition to genistein, treatment with tyrphostin A48, a specific inhibitor for EGFR PTK, caused an increase in the numbers of blue cells. These results suggest that EGF-R PTK may be involved in recombinant AAV-mediated transgene expression (Mah et al., 1998).

In order to further investigate the role of EGFR PTK in recombinant AAV transduction efficiency, other inhibitors specific for EGFR PTK, tyrphostins 1, 23, 25, 46, 47, 51, 63, and AG1478 (Yaish et al., 1988; Gazit et al., 1989; Lyall et al., 1989; Levitzki, 1990, Levitzki et al., 1991) were tested for their effects on recombinant AAV

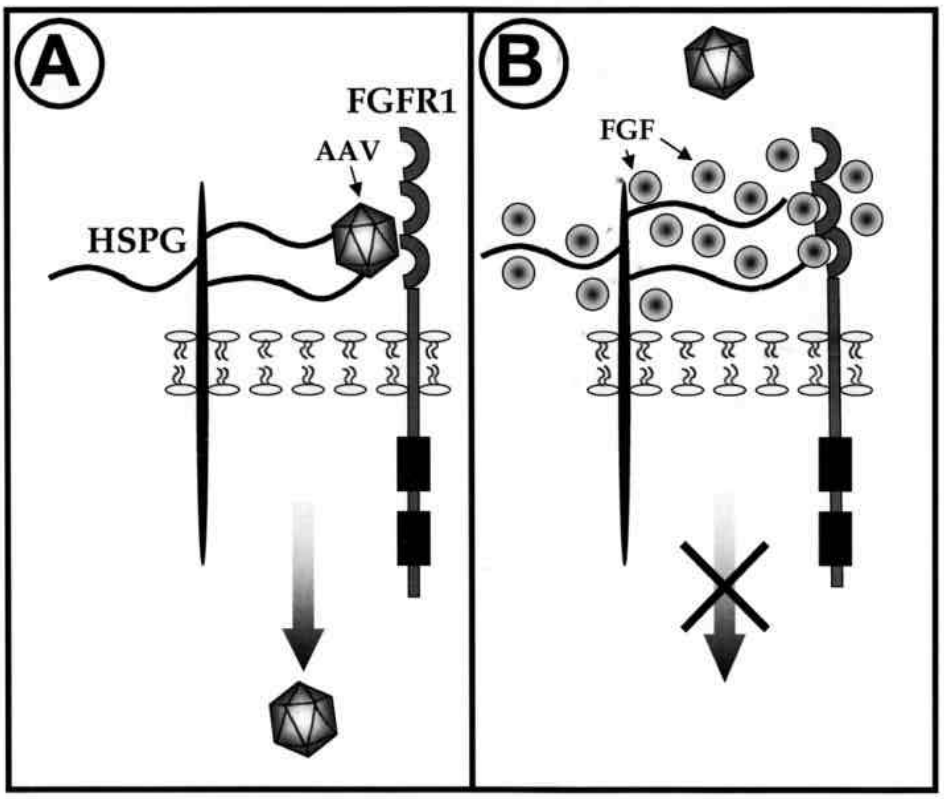

Figure 1. A possible model for the role of cell surface HSPG and FGFR1 in mediating AAV binding and entry into the host cell. Co-expression of HSPG and FGFR1 is required for successful binding of AAV followed by viral entry into a susceptible cell (**Panel A**), both of which are perturbed by the ligand, FGF, which also requires HSPG-FGFR1 interaction (**Panel B**) (Qing et al., 1999).

transduction. For controls, tyrphostins specific for tumor necrosis factor α (TNF-α) production, AG126, TNF-α cytotoxicity, AG1288 (Novogrodsky et al., 1994), platelet-derived growth factor receptor protein tyrosine kinase (PDGFR PTK), AG1295 and AG1296 (Kovalenko et al., 1994), were also used. It was evident that among all the inhibitors tested, treatment with tyrphostin 1 resulted in the greatest increase in recombinant AAV transduction efficiency (without causing significant cytotoxicity) followed by that of tyrphostins 23, 63, 25, 46, then 47. Again, these results emphasize the role EGFR PTK plays in recombinant AAV-mediated transgene expression. As expected, the control tyrphostins AG126, AG1288, AG1295, and AG1296 had no significant effect. In toxicity experiments, with reference to the mock-treated or solvent alone controls, both tyrphostin 1 and tyrphostin 23 are far less toxic than either genistein or hydroxyurea (HU), two reagents that have been previously shown to increase AAV transduction efficiency (Russell et al., 1995; Ferrari et al., 1996; Qing et al., 1997). Therefore, treatment of primary cells with tyrphostin may offer a physiological means to increase recombinant AAV transduction efficiency without causing deleterious effects (Mah et al., 1998).

We have previously demonstrated that recombinant AAV transduction efficiency correlates well with the phosphorylation state of the cellular ssD-BP (Qing et al., 1998). For example, in HeLa cells, the ssD-BP is predominantly in the phosphorylated form, and these cells are not readily transduced by recombinant AAV vectors. 293 cells, on the other hand, are very well transduced by recombinant AAV, and have been demonstrated to contain predominantly the dephosphorylated form of the ssD-BP. Following treatment of HeLa cells, all active tyrphostins caused a significant increase in the amount of dephosphorylated form of the ssD-BP, as determined by electrophoretic mobility-shift assays (EMSAs). Consistent with our previous data (Qing et al., 1998), the amount of dephosphorylated ssD-BP for each treatment corresponded with the level of increase in transduction efficiency for each of the compounds. That is, the greater the amount of dephosphorylated ssD-BP, the greater the increase in AAV-mediated transgene expression. When 293 cells, either mock-treated or treated with EGF, were analyzed, the ssD-

BP was present mostly in the dephosphorylated form in mock-treated cells as observed previously (Qing et al., 1997, 1998), whereas EGF treatment resulted in a significant increase in the amount of the phosphorylated form of ssD-BP. These results strongly suggest that EGFR PTK plays a direct role in the phosphorylation of the ssD-BP (Mah et al., 1998).

B. Recombinant AAV transduction efficiency correlates inversely with the EGFR expression

If EGFR PTK is responsible for catalyzing phosphorylation of the ssD-BP, then AAV-mediated transgene expression would be expected to be significantly lower in cells which express higher numbers of EGFRs than those which express fewer numbers of EGFRs. Therefore, AAV transduction efficiency would inversely correlate with the extent of EGF-R expression. To further investigate this hypothesis, equivalent numbers of cells known to express very high numbers of EGFRs, A431 cells (Giard et al., 1973), and cells known to express very low numbers, H69 cells (Gamou et al., 1987), in addition to HeLa and 293 cells, were infected with a recombinant AAV vector under identical conditions followed by X-gal staining 48 hrs post-infection. Consistent with previously published data (Qing et al., 1998), the transduction efficiency in HeLa and 293 cells was approximately 4% and 20%, respectively. As expected, the transduction efficiency in A431 cells was less than 1%. Contrary to our hypothesis, very little transduction (<1%) was also noted in the H69 cells. This apparent paradox was addressed by performing radiolabeled EGF and AAV binding assays. EGF binding assays demonstrated that A431 cells bound the greatest amounts of EGF, followed by HeLa, then 293 cells. H69 cells bound negligible amounts of EGF, as expected. It was evident from AAV binding assays that, similar to M07e cells, previously shown to lack AAV receptors (Bartlett and Samulski, 1998), H69 cells also do not express the cellular receptor for AAV. On the other hand, the low transduction efficiency seen in the A431 cells could not be attributed to a lack of expression of AAV receptors as these cells expressed far greater numbers of AAV receptors than HeLa or 293 cells.

As the EGFR PTK appeared to catalyze the phosphorylation of the ssD-BP, it was next of interest to examine the effects of EGF, as well as tyrphostin- and genistein-treatments on A431 and H69 cells. Due to the high-levels of expression of EGFR in A431 cells, we hypothesized that the ssD-BP would be present in its phosphorylated form and that EGF treatment would have no effect on it's phosphorylation state. Similarly, it would be expected that H69 cells would also fail to respond to EGF treatment since little expression of the EGFR occurs in these cells. To this end, equivalent numbers of A431 and H69 cells were either mock-treated or treated with EGF and analyzed by EMSA. As expected, EGF-treatment had no significant effect on the phosphorylation state of the ssD-BP in either cell type. In A431 cells, the ssD-BP was found to be predominantly in the phosphorylated form due to the high-levels of EGFR PTK expression. On the other hand, both phosphorylated and dephosphorylated forms of the ssD-BP were detected in H69 cells. Interestingly, however, treatment with either tyrphostin or genistein resulted in the conversion from the phosphorylated to the dephosphorylated form of the ssD-BP, consequently resulting in increased transduction in A431 cells. Under identical conditions, however, neither tyrphostin nor genistein treatments had any effect on the phosphorylation state of the ssD-BP in H69 cells, and these cells could not be transduced by AAV as they lack the cell surface receptor for AAV. Although it may not be readily apparent which cellular protein tyrosine kinase is responsible for the phosphorylation of the ssD-BP in H69 cells, these results are in agreement with the conclusion that the phosphorylation of the ssD-BP in A431 cells is catalyzed by the EGFR PTK (Mah et al., 1998).

C. Stable transfection of EGFR cDNA into 293 cells causes phosphorylation of the ssD-BP and results in inhibition of AAV-mediated transgene expression

As 293 cells can be efficiently transduced by recombinant AAV vectors, since they contain predominantly the dephosphorylated from of the ssD-BP (Qing et al., 1997, 1998), we examined whether the deliberate over-expression of EGFR PTK in these cells would lead to phosphorylation of the ssD-BP, and consequently, result in the inhibition of AAV-mediated transgene expression. 293 cells were transfected with an EGFR cDNA expression plasmid and a number of stably transfected clones were used to determine the ratios of the dephosphorylated to the phosphorylated forms of the ssD-BP and compared with that in control, untransfected 293 cells. Replicate cultures were also evaluated for the efficiency of recombinant AAV transduction, with or without pre-treatment with tyrphostin 1. These results indicated that in each of the transfected 293 cell clones, the ratio of dephosphorylated/phosphorylated ssD-BPs was reduced to an average of 0.45 from greater than 3.5 in control cells, which concomitantly led to a significant decrease in AAV transduction efficiency from approximately 18% in control 293 cells to an average of about 2% in the EGF-R-transfected 293 cell clones. These data strongly support the hypothesis that the EGFR-ssD-BP interaction plays a crucial role in AAV-mediated transgene expression.

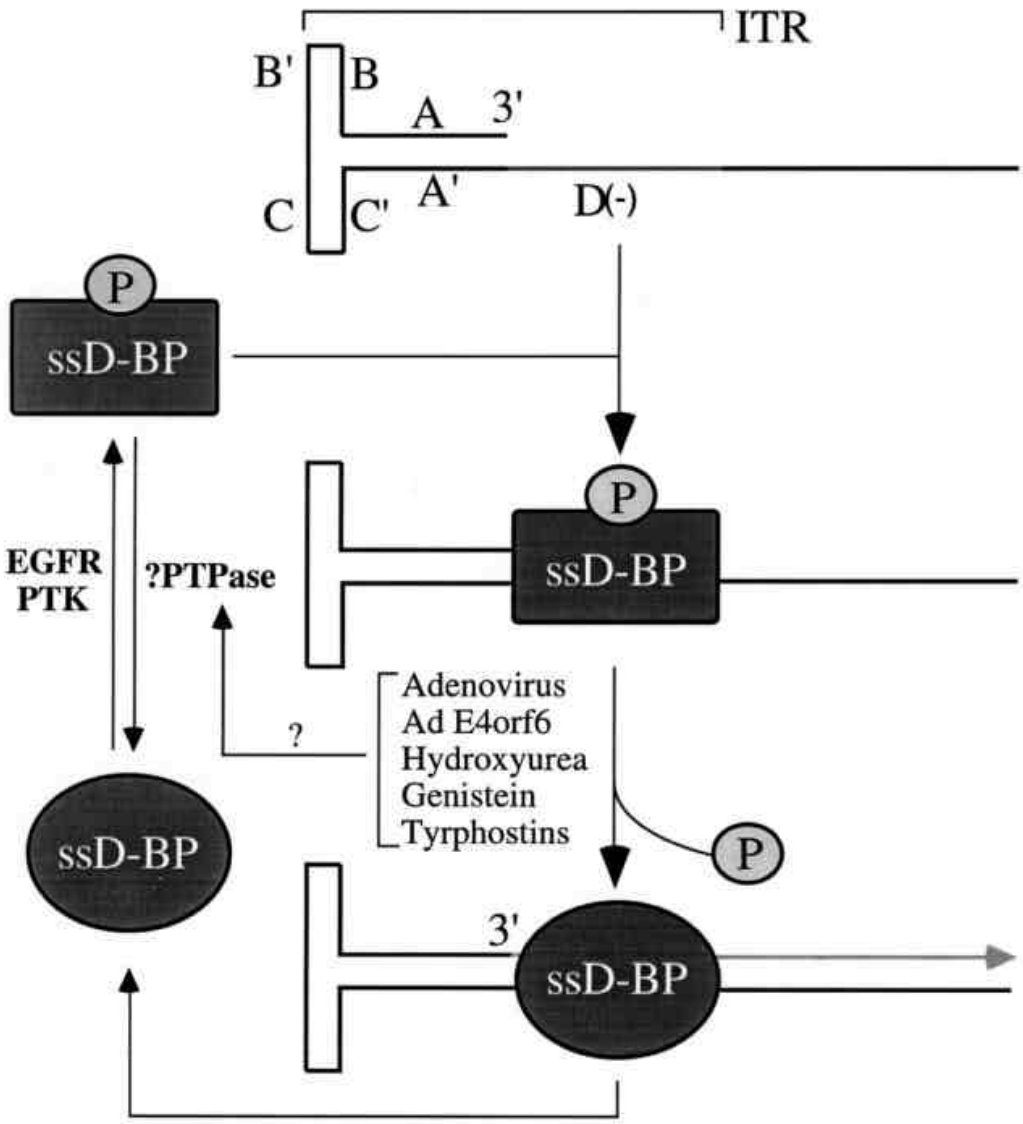

Figure 2. A possible model for the role of the cellular EGFR PTK in AAV-mediated transgene expression. The phosphorylated ssD-BP, which is phosphorylated by the EGFR PTK, binds to the single-stranded D-sequence within the AAV-ITR, and blocks the viral second-strand DNA synthesis. Co-infection with adenovirus, or expression of the Ad E4orf6 protein, or treatment with HU, genistein, or tyrphostins, leads to dephosphorylation of the ssD-BP, either via inhibition of the EGFR PTK, or via activation of a hitherto unknown cellular phosphotyrosine phosphatase, leading to some type of conformational change in the ssD-BP which, in turn, allows the viral second-strand DNA synthesis resulting in augmentation in transcription and translation of the transgene (Mah et al., 1998).

Subsequent *in vitro* phosphorylation assays performed with the commercially available purified EGFR PTK (McGlynn et al., 1992; Weber et al., 1984) and the affinity column-purified dephosphorylated form of the ssD-BP from 293 cells indicated that the ssD-BP was phosphorylated by the EGFR PTK and that this phosphorylation was abrogated in the presence of tyrphostin 1 and tyrphostin 23. These results provide direct evidence that the ssD-BP is a downstream target of the EGFR PTK. Based on all the available data, we propose a model for the subsequent steps in AAV-mediated transduction which is shown in **Figure 2**. In this model, the phosphorylated ssD-BP, which is phosphorylated by the EGFR PTK, binds to the single-stranded D-sequence within the AAV-ITR, and blocks the viral second-strand DNA synthesis. Co-infection with adenovirus, or expression of the Ad E4orf6 protein, or treatment with HU, genistein, or tyrphostins, leads to dephosphorylation of the ssD-BP, either via inhibition of the EGFR PTK, or

via activation of a hitherto unknown cellular phosphotyrosine phosphatase, leading to some type of conformational change in the ssD-BP which, in turn, allows the viral second-strand DNA synthesis resulting in augmentation in transcription and translation of the transgene (Mah et al., 1998).

IV. Conclusions and future prospects

The identification of FGFR1 as a co-receptor for AAV is an important step forward (Qing et al., 1999). Interestingly, however, although FGFRs have been shown to be expressed in every organ and tissue examined (Givol and Yayon, 1992), the relative abundance of their expression in skeletal muscle and in neuroblasts and glioblasts in the brain correlates particularly well with the documented high efficiency of AAV-mediated transduction in these tissues *in vivo* (Fisher et al., 1997; Kaplitt et al., 1994; Kessler et al., 1996; McCown et al., 1996; Xiao and Samulski, 1996). Since there are at least four distinct but related members in the FGFR family, viz FGFR1, FGFR2, FGFR3, and FGFR4 (Ledoux et al., 1992), it should be of interest to now systematically examine, both *in vitro* and *in vivo*, the relative involvement of each of these members in facilitating successful infection by AAV.

The demonstration that the cellular EGFR PTK catalyzes phosphorylation of the ssD-BP, a crucial player in AAV-mediated transduction, this kinase should be an easy target for inhibition by low-toxicity compounds for their ability to significantly increase recombinant AAV transduction efficiency which may prove to be valuable for gene therapy. Although it is possible that other factors, in addition to the ssD-BP phosphorylation state, act in concert to influence the AAV transduction efficiency, it is noteworthy, however, that skeletal muscle and brain tissues, which have been shown to be extremely well-transduced by recombinant AAV vectors *in vivo* (Fisher et al., 1997; Kessler et al., 1996; Kaplitt et al., 1994; McCown et al., 1996; Xiao and Samulski, 1996), express little to no EGFR (Lim and Hauschka, 1984; Styren et al., 1993). Further studies on the interaction between FGFR and additional downstream target proteins, and the possible interaction between FGFR and EGFR should allow for a clearer understanding of molecular events involved in high-efficiency AAV transduction which, in turn, should lead to improvements in the optimal use of AAV vectors in human gene therapy.

Acknowledgments

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