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# Capsid-Incorporation of Antigens into Adenovirus Capsid Proteins for a Vaccine Approach

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## Abstract

Some viral vectors are potent inducers of cellular and humoral responses; therefore, viral vectors can be used to vaccinate against cancer or infectious diseases. This report will focus on adenovirus (Ad)-based vectors. Traditional viral-vector vaccination embodies the concept that the vector uses the host-cell machinery to express antigens that are encoded as transgenes within the viral vector. Several preclinical successes have used this approach in animal model systems. However, in some instances, these conventional Ad-based vaccines have yielded suboptimal clinical results. These suboptimal results are ascribed, in part, to preexisting Ad serotype 5 (Ad5) immunity. To address this issue, the “antigen capsid-incorporation” strategy has been developed to circumvent the drawbacks associated with conventional transgene expression of antigens by Ad vectors. This strategy embodies the incorporation of antigenic peptides within the capsid structure of viral vectors. Incorporating immunogenic peptides into the Ad capsid offers potential advantages. Importantly, vaccination by means of the antigen capsid-incorporated approach results in a strong humoral response, similar to the response generated by native Ad capsid proteins. This strategy also allows for the boosting of antigenic specific responses. This strategy may be the way forward for improved vaccine schemes, especially for those infections requiring a strong humoral antigenic response.

### Keywords

Vaccine; Antigen; Capsid-Incorporation; Adenovirus; Virus

## Introduction

Some viral vectors are potent inducers of cellular and humoral responses; therefore, using recombinant viral vectors expressing tumor antigens to activate the immune system is a promising approach to prevent or treat cancer. In addition, viral vectors can be used to express protein from pathogens to vaccinate against infectious diseases. Several viral vectors have been successfully used for vaccination in experimental models. Some viral vectors that have been used for vaccination against cancer and infectious diseases include: alphaviruses, human rhinoviruses, adenoviruses (Ads), picornaviruses, poxviruses, measles viruses, and influenza viruses [1–12]. Although each of these vectors has disadvantages and advantages with respect to vaccine development, this report will focus on those of Ad-based vectors.

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## Adenovirus Vectors as Vaccine Agents

In 2009, Ad-based vectors accounted for 23.9% of those in gene-therapy clinical trials[13]. The broad utility of these vectors is derived from several key characteristics: (a) the recombinant viral genome is readily manipulated; (b) replication-defective Ads can be propagated in complementing cell lines; (c) Ads infect a broad range of target cells [14,15]; and (d) Ads can achieve high levels of in vivo gene transfer with concomitantly high levels of transgene expression [15,16]. These concepts have also led to Ads being used as molecular vaccine agents.

Traditional viral-vector vaccination embodies the concept that the vector uses the host-cell machinery to express antigens that are encoded as transgenes within the viral vector. Cellular and humoral immune responses are generated against these antigens for a vaccine effect. Several preclinical successes have used this approach in animal model systems. However, in some instances, these conventional Ad-based vaccines have yielded suboptimal clinical results. These suboptimal results are ascribed, in part, to preexisting Ad serotype 5 (Ad5) immunity (vector liver sequestration is another major drawback, however; this will not be discussed in this review). It is estimated that 50% to 90% of the adult population has preexisting immunity (PEI) to Ad5; if one of these Ad5-immune individuals is vaccinated with an Ad vector for therapeutic purposes, there may be limited transgene or antigen expression due to Ad clearance by the immune system [17–21].

The “antigen capsid-incorporation” strategy has been developed to circumvent the drawbacks associated with conventional transgene expression of antigens by vectors. With respect to Ad, one drawback to conventional transgene expression of antigen is the inability of Ad-based vectors to produce a potent humoral response against certain antigens (as seen in the case of some malaria antigens) [22]. In addition, this strategy may allow vectors to circumvent Ad5 PEI yielding a more robust immune response to either the antigen presented on the vector capsid or the antigen that is expressed as a transgene. The antigen capsid-incorporation strategy embodies the incorporation of antigenic peptides within the capsid structure of viral vectors. Incorporating immunogenic peptides into the Ad capsid offers potential advantages. Importantly, processing the capsid-incorporated antigen via the exogenous pathway could result in a strong humoral response, similar to the response generated by native Ad capsid proteins. In addition, because anti-Ad capsid responses are amplified by administering the vector repeatedly, immune responses against antigenic epitopes that are part of the Ad capsid should be increased by this approach as well, thus allowing boosting of the response [23–25]. This strategy may have additional benefits of promoting immune cross-priming [26,27] and activation of CD8+ T cells by means of incorporating T cell helper epitopes into the Ad capsid proteins [28]. Therefore, this novel antigen capsid-incorporation approach may offer exciting opportunities to create Ad-based vaccine vector strategies that circumvent the major limitations associated with Ad vectors.

## Adenovirus Capsid Structure

With respect to Ad, the ability to circumvent normal host anti-vector immunity has been accomplished by genetically modifying the Ad capsid. This capacity has been greatly fostered by the intrinsic plasticity of the adenoviral capsid proteins [29,30].

The Ad capsid protein has 4 distinct domains available for antigen incorporation; these include: the hexon (polypeptide II), penton base (polypeptide III), fiber (polypeptide IV), and polypeptide IX (pIX)[31] (Figure 1)[32].

Hexon is the most abundant of the capsid’s structural proteins, accounting for 63% of the total protein mass [33,34]. Ad2’s hexon polypeptide is 967 amino acids; the longest known molecular design of a hexon. Early analysis of the protein sequences of different hexon

proteins revealed that, in addition to the conserved regions, there were 7 discrete hypervariable regions (HVRs) (now reclassified as 1–9). These HVRs do not appear to be involved in binding any other viral proteins [35]. The HVRs of hexon contain serotype-specific epitopes [34,35]. The loops at the top of the HVRs are the most amenable to modification by genetic engineering. Several groups have shown that short heterologous peptides can be incorporated within the HVRs of the hexon without affecting the virion's stability or function. Of note, a subset of these modifiable loops were exposed on the surface of the capsid [36,37]. HVR2 and HVR5 are the most flexible with respect to peptide or antigen incorporation [28,36–41]. These studies highlight the high level of plasticity of hexon protein and suggest that additional vaccine utilities may be derived by exploiting these Ad capsid flexibilities.

Early electron microscopy (EM) studies of the penton base (a pentamer of polypeptide III) revealed that, the penton base has a polygonal cross-section with a central diameter having an approximately 30-Å diameter. There are approximately 571 residues of penton monomer. The penton base and fiber (a trimer of polypeptide IV) form the penton complex that seals the capsid protein at its 12 vertices. The fiber is responsible for attachment to the host cell, and the penton base is responsible for internalization of the virus. A series of complex events trigger membrane permeabilization and virus internalization. In brief, during the virus-entry process, the penton base binds $\alpha_v\beta3$ integrins. The binding of penton base to $\alpha_v\beta3$ integrins promotes virus infection by stimulating internalization into clathrin-coated vesicles rather than enhancing virus attachment [42–44]. This interaction has been demonstrated for 4 adenovirus subgroups (A,B,C, and E), and is attributed to a conserved Arg-Gly-Asp (RGD) motif within a variable region of the penton base [45–48].

The penton base has been used for antigen incorporation strategies. The fiber is shed from the Ad capsid after internalization, exposing the penton base-modified Ad more efficiently to the host and allowing an immune response to be mounted [49,50]. However, there have been very few attempts to incorporate peptides or antigen within the penton base [41]. This fact may be due to the structural constraints related to antigen incorporation at the base of the capsid.

The fiber protein protrudes from 12 vertices of the capsid surface of the virion. Fiber exists as a trimeric structure composed of 3 domains: an N-terminal tail that attaches to the penton base, a central shaft with repeating motifs, and a C-terminal globular "knob" domain that is responsible for virus attachment to the host cell. With respect to Ad5, its fiber knob primarily binds the coxsackie-adenovirus receptor (CAR). The knob 3 structure of Ad3 [51](a non-CAR binder) is similar to those of Ad5 [52], Ad2 [53], and Ad12 [54]. A wide range of variations exist in fiber length among the Ad serotypes because of the differences in the number of repeats in the fiber shaft, ranging from 6 residues in Ad3 [55] to 22 residues in Ad2 and Ad5 [56]. With respect to fiber modifications, several groups have incorporated heterologous peptide motifs at the carboxy terminus and HI loop of fiber. These groups have also developed a more radical strategy to replace fiber with a substitute trimerization domain, allowing capsid incorporations of greater size and complexity [57–60].

One of the most characterized Ad minor capsid proteins is pIX. There are approximately 240 copies of this protein per virion, and each copy acts as a cement protein binding a group of nine hexons in each facet of the capsid [61]. In addition to stabilizing the capsid structure, pIX promotes efficient virus proliferation. This protein has been a promising target for genetic manipulation; small and large epitopes have been incorporated into its C-terminus for imaging studies, cell targeting, and vaccination strategies [36,41,62–73].

## Incorporation of Antigens into Viral Capsid Structures for a Vaccine Approach

Incorporating antigens into viral capsid structures has been used as a vaccination approach for several diseases [28,38,40,41,74]. One of the first instances in which this strategy was used was in research performed by Crompton and colleagues in 1994. They genetically incorporated an 8 amino-acid sequence of the VP1 capsid protein of poliovirus type 3 into 2 regions of the adenovirus serotype 2 hexon. One of the chimeric vectors produced by using this method grew well in tissue culture, and antiserum raised against the Ad with the polio antigen specifically recognized the VP1 capsid of polio [74].

Similar studies have been performed by other research groups. For example, Worgall and colleagues used the antigen capsid-incorporation strategy to vaccinate against *Pseudomonas aeruginosa* (*pseudomonas*), a gram-negative bacterium that causes respiratory tract infections in individuals who have cystic fibrosis or are immunocompromised[75] . Since *pseudomonas* is an extracellular pathogen, anti-*pseudomonas* humoral immunity should be sufficient to provide protective immunity. Thus, *pseudomonas* is a candidate for vaccine development. Several immunogenic peptides have been identified in the outer membrane protein F (OprF) of *pseudomonas*, including the immunodominant 14-mer peptide Epi8. This study describes genetic incorporations of a neutralizing epitope from the *pseudomonas* Epi8 into Ad5 HVR5 (AdZ.Epi8) [28]. BALB/c mice vaccinated with the capsid-modified vectors showed an increase in antibody response consisting of both anti-*pseudomonas* IgG1 and IgG2a subtypes. Additionally, after mice immunized with the virus containing the OprF epitope were subjected to pulmonary challenge with *pseudomonas*, 60% to 80% of them survived.

The same group performed another study in which they constructed an optimized vaccine vector, AdOprF.RGD.Epi8. The 2 capsid modifications of the AdOprF. RGD.Epi8 vector included genetic incorporations of RGD into the fiber to enhance the infection of cells dendritic cells (DCs) [76,77] as well as the insertion of the 14-mer OprF epitope Epi8 into HVR5 of the Ad hexon to enable repeat administration of the same vector to boost the anti-OprF humoral response [76,77]. Worgall's study demonstrates that AdOprF.RGD.Epi8 expresses OprF, contains Epi8 in the hexon protein, and enhances gene transfer to dendritic cells more than AdOprF does (a similar Ad vector that expresses OprF with an unmodified capsid). Intramuscular immunization of C57BL/6 mice with AdOprF.RGD.Epi8 resulted in the generation of anti-OprF antibodies at similar levels to those induced by immunization with AdOprF; however, immunization with AdOprF.RGD.Epi8 was associated with increased CD4+ and CD8+ gamma interferon T-cell responses against OprF. The RGD-modified vector enhances expression of integrin-expressing cells, such as dendritic cells, and enhances the immune response generated against transgenes in animal model systems [76,77]. Most importantly, immunization of mice with AdOprF.RGD.Epi8 increased their survival rates after pulmonary challenge with lethal doses of *pseudomonas*. Importantly, repeat administration of AdOprF.RGD.Epi8 boosted the humoral anti-OprF response and increased protection, whereas no boosting was achieved by repeat administration of AdOprF. These data suggest that the capsid-modified AdOprF.RGD.Epi8 vector is a more effective immunogen than the wild-type Ad capsid is, making this modified vector a good candidate for use in an anti-*pseudomonas* vaccine. This strategy whereby antigen-presenting cells are activated to elicit a robust immune response to capsid-incorporated antigens is a novel approach and requires extensive investigation.

In contrast to these results, McConnell et al., found that chimeric hexons containing incorporations of *B anthracis*' protective antigen (PA) elicited antibodies against PA in mice but failed to yield protection against a challenge with anthrax toxin, lethal factor (*B. anthracis* secretes three subunits that make up the anthrax toxins, PA, lethal factor, and edema factor) [78]. They speculated that the varying results could be a result of a variety of

things. For instance, this lack of toxin neutralization could have been a result of inadequate neutralizing antibodies (Nab) generated by the immunization schedule. The authors also speculate that difference seen in their study compared to Worgall and colleagues could represent either a difference in the ability of the selected epitopes to elicit a Nab response in two different disease models or a difference in the antibody titers necessary to achieve protection against *pseudomonas* compared to those needed to protect against lethal factor challenge. In addition, the authors speculated that the latter may be related to the fact that, in the anthrax model, the response is directed against a secreted bacterial toxin, and in the *pseudomonas* model, the response is directed against the bacterium itself[38].

Similar studies have been performed by Krause et al., [41]. The focus of their study was determining which of the capsid proteins could be modified without affecting the infectivity of the Ad and while still inducing high anti-epitope immunity. This study compared the immune response generated by incorporating the hemagglutinin (HA) protein of the influenza A virus into the outer Ad capsid proteins: hexon, penton base, fiber knob, or pIX. The HA epitope was recognized by the anti-HA antibody in all 4 of the modified virions, although binding to the HA presented in hexon HVR5 was slightly stronger. It is a logical assumption that that genetic incorporation of antigens within the hexon protein would yield the most anti-epitope immune response because the hexon protein is more abundant than any other capsid protein. Of note, immunizations of mice with either the same Ad particle numbers, resulting in different amounts of HA copy numbers, or the same amount of HA copies, resulted in the highest humoral and cellular responses from the vector that contained HA within the fiber knob. As previously mentioned, fiber is approximately 60 times less abundant than hexon is on the Ad virion, and pIX is approximately 3 times less abundant than hexon is on the Ad capsid. These data suggest that, in this model system, antigenic epitopes that are incorporated within the fiber knob elicit the optimal response from an Ad-based vaccine. However, this study did not investigate whether or not the size of the incorporated epitopes also affected the immune response generated.

To expand on knowledge gained from previous antigen capsid-incorporation studies, our group set out to create novel vaccine vectors that would yield optimal vaccine efficacy. Therefore, our focus was to develop vectors that could aid in the development of multivalent vaccine vectors. Our 2008 manuscript explored the use of either Ad5 HVR2, HVR5, or both to create vectors containing identical antigenic epitopes in either region. To compare the flexibility and capacities of Ad5 HVR2 to those of HVR5, we genetically incorporated identical epitopes of increasing size within HVR2 or HVR5 of the Ad5 hexon. These epitopes ranged in size from 33–83 amino acids. Stable viruses were produced with incorporations of 33 amino acids plus a 12 amino acid linker at HVR2 or HVR5. In addition, stable viruses were produced with incorporations up to 53 amino acids plus a 12 amino acid linker in HVR5. With respect to the selected antigen incorporations in Ad5 HVR2 or HVR5, HVR5 was more permissive, allowing an epitope incorporation of 65 amino acids. Whole virus ELISA analysis revealed that these model antigens were surface-exposed, and in vivo immunization with these vectors elicited antigen-specific immune responses [40].

Recently, Shiratsuchi, et. al, tested the antigen capsid-incorporation strategy to develop a vaccine against malaria. In an effort to enhance humoral response to *Plasmodium yoelii* circumsporozoite (CS) protein and circumvent Ad vector PEI, they constructed and analyzed various vectors containing the CS protein of malaria. These vectors contained the CS protein in the hexon and/or fiber region of the Ad capsid as well expressed the CS protein as a transgene. Several vaccinations with the antigen capsid-modified vectors induced a substantially increased level of protection against subsequent malaria challenge in mice when compared with unmodified WT/CS-GFP. However, in brief they illustrated that CS incorporation at HVR1, but not other portions of the capsid proteins, could circumvent Ad

PEI, while maintaining the immunogenicity of a B cell epitope of the CS protein expressed on the capsid and expressed as a transgene. This is an important finding because of two reasons: this is the first published report of the antigen capsid-incorporation strategy for malaria and this is the first report of antigen incorporated into Ad HVR1 for vaccine purposes [22].

In 2010, Bayer and colleagues constructed an Ad vector that could be used to vaccinate against Friend virus (FV), a murine virus that causes robust polyclonal erythroblast proliferation, leading to spleen enlargement, erythroleukemia, and death in adult mice [79]. The FV infection model allows the study of retrovirus infection in a model with similarities to HIV [80]. Using the FV model, Bayer's group evaluated a vaccine strategy that combined genetic and protein vaccination by using an Ad vector that vaccinated by means of transgene antigen expression and capsid-incorporation of antigen (Ad antigen expression/ antigen display vector). This study by Bayer, et. al, demonstrates that the Ad antigen expression/ antigen display vector lead to better protection against FV challenge as compared to (a) conventional antigen expression vector or (b) antigen display-only vector. In this regard, this improved protection correlated with neutralizing antibody levels and CD4+ responses. Using a vector that displays gp70 (without encoding it), they found that antigen display on the capsid alone was sufficient to induce high levels of binding antibodies. However, for the induction of neutralizing antibodies, the display on the particle alone was not sufficient. They speculated that antigen expression by *in vivo* transduction of the Ad antigen expression/antigen display vector lead to increase Nab response and CD4+ T cell responses.

Bayer and group speculate that there are several advantages to pIX incorporation as compared to incorporation in hexon, fiber, or penton base, which have been previously explored for antigen presentation [28,39–41,74]. For example, pIX allows large proteins to be incorporated with minimal loss of function or viral integrity [64–67,69,71]. These researchers also speculate that whole-protein incorporation is a promising approach to capsid-incorporation; the presence of several relevant epitopes due to expression of a whole protein would allow for a broader vaccine application. Furthermore, displaying an entire protein rather than just the antigenic epitopes on the vector surface may be advantageous because the B-cell epitopes would be more likely to be in the proper confirmation when incorporated as part of the original protein rather than as a scaffold protein. This problem was encountered when incorporating polio virus epitopes within hexon protein [74]. Additional advantages of pIX display include its arrangement into trimers on the Ad capsid surface [81]; antigens presented on pIX would be in close proximity to one another and may be able to form trimers on the Ad capsid, possibly allowing for the induction of conformation-dependent antibodies. Similar results were obtained by Boyer et al.; they showed that Ad vectors displaying Y. *pestis'* V antigen or F1 capsular antigen on the virion surface elicit a higher V- or F1-specific antibody response, allowing boosting and better protection against a lethal challenge than that produced by vaccination with V or F1 proteins that are paired with conventional adjuvants [82].

## Antigen Capsid-Incorporation for HIV Vaccination

Since its discovery and characterization in the early 1980s, there have been several advancements as scientists continue to search for ways to eradicate HIV from the human population. The majority of the effort and nearly all of the success have come in the area of patient treatment rather than in inhibiting contraction or spread of the virus. However, treatments are often costly and disruptive to the daily lives of patients. Therefore, a safe and effective HIV vaccine is urgently needed.

Abe and colleagues evaluated the ability of Ad5-based vectors expressing an HIV transgene to induce antigen-specific immune responses under Ad5 pre-immune conditions. To

overcome limitations that are generally experienced because of pre-existing immunity to Ad5, they constructed vectors that have a modification in the HVR5. Their study, characterized various immunological parameters generated by these vectors such as vector neutralization, acquisition of adaptive immune response, and comparison of protective immunity. First, in order to evaluate the utility of the modified Ad vector, they measured the neutralizing activity of sera by a modified-Ad vector. They administered Ad-Luc (luciferase protein expressed as a transgene in the Ad E1 deleted region), Ad-HisLuc ($His_6$ epitope presented in the HVR5 region and luciferase protein expressed as a transgene in the E1 deleted region), or Ad-END/AAALuc vector (containing 3 amino-acid mutations in HVR5 and expressing luciferase protein in the E1 deleted region) to mice intramuscularly. After administration of these vectors, neutralizing activity against Ad5 was observed from 0–8 weeks. The hexon-modified vector (i.e., Ad-HisLuc) generated the lowest Ad5-specific neutralizing activity, which was significantly lower than that generated by Ad-Luc at weeks 6 and 8 or by Ad-End/AAALuc at week 8. The individual neutralizing activity of Ad-HisLuc immunization was significantly lower than that of Ad-Luc immunization. Studies performed by this research group indicate that a change in the immunogenic epitope is necessary to avoid neutralization by pre-existing Nabs. Additional studies performed by Abe and colleagues support the concept that modified hexon inhibits Ad5 NAbs and promotes cellular immune responses by inducing antigen-specific immune responses after transgene expression [83].

Our most recent study exploits the antigen capsid-incorporation strategy further by using novel vectors that were constructed to provide cellular and humoral HIV immunity [84]. Our study is the first of its kind to genetically incorporate an HIV antigen within the Ad5 hexon's HVR2 alone or in combination with the genomic incorporation of a Gag transgene (Ad5/HVR2-MPER-L15(Gag)). In this study, we successfully incorporated a 24 amino-acid epitope of HIV within HVR2. The HIV region selected for HVR2 incorporation was the membrane proximal ectodomain region (MPER) derived from HIV glycoprotein (gp)41. When the MPER epitope was incorporated into HVR2 in combination with transgene expression, we observed growth kinetics and thermostability changes similar to those observed in other studies after using capsid-incorporated vectors, [67,85] indicating that incorporation of the MPER epitope within HVR2 was not dramatically detrimental to virus characteristics [66,85]. In addition, we demonstrated that the MPER epitope is surface-exposed within HVR2. Most importantly, we observed a humoral anti-HIV response in mice vaccinated with the hexon-modified vector. The MPER-modified vector allows boosting compared to AdCMVGag, possibly because the Ad5/HVR2-MPER-L15(Gag) Ad elicits less of an anti-Ad5 immune response. It is possible that the MPER epitope reduced the immunogenicity of the Ad5 vector. This finding is noteworthy because HVR2 has not been fully explored for use in antigen capsid-incorporation strategies.

With respect to HIV vaccination, the antigen capsid-incorporation strategy has also been evaluated within the context of human rhinovirus vectors. Research groups have constructed human rhinovirus:HIV chimeras to stimulate immunity against HIV-1 [4,86]. Additionally, researchers have generated combinatorial libraries of human rhinovirus capsid-incorporated HIV-1 gp41 epitope, eliciting antibodies whose activity can mimic the Nab effect [4,86].

Commercial and clinical development of Ad-based HIV vaccines have progressed more than the development of vector systems such as human rhinovirus because the flexibility of Ad generally exceeds that of current rhinovirus systems. For example, because human rhinovirus is a relatively small RNA virus, the human rhinovirus platform can only display 60 copies of a single HIV-1 epitope [4,86]. In contrast, the Ad vector capsid platform could allow incorporation of HIV-1 epitopes into 4 structurally distinct domains including: hexon

(HVR2 and HVR5)[83], fiber, penton base, and pIX, similar to the illustration depicted in Figure 1.

In comparison, the Ad MPER antigen capsid-incorporation display platform can present an array of 720 HIV-1 epitope copies within Ad hexon and 240 HIV-1 epitope copies within pIX. If a multivalent Ad vector is generated with HIV epitopes within the hexon and pIX domains this would represent 960 HIV epitopes within one Ad particle. Another significant difference between the Ad and human rhinovirus platforms is in the number of locales that have been successfully used to insert heterologous epitopes. Lastly, in contrast to the rhinoviruses, which lack this capacity, the Ad platform has sufficient coding capacity, allowing for HIV-1 transgene expression and presentation of either the same or a different antigen on the viral capsid surface. This latter finding is important because it provides the basis for constructing vectors that will provide both cellular and humoral HIV immunity potentially leading to a prophylactic HIV vaccine. Based on setbacks and progress from recent HIV clinical trial findings, there has been a shift in the vaccine paradigm. There is a urgent need for vectors that provide both cellular and humoral HIV immunity. Vectors and strategies described in this review could potentially be the way forward for safe and effective HIV vaccination as well as vaccination for other diseases.

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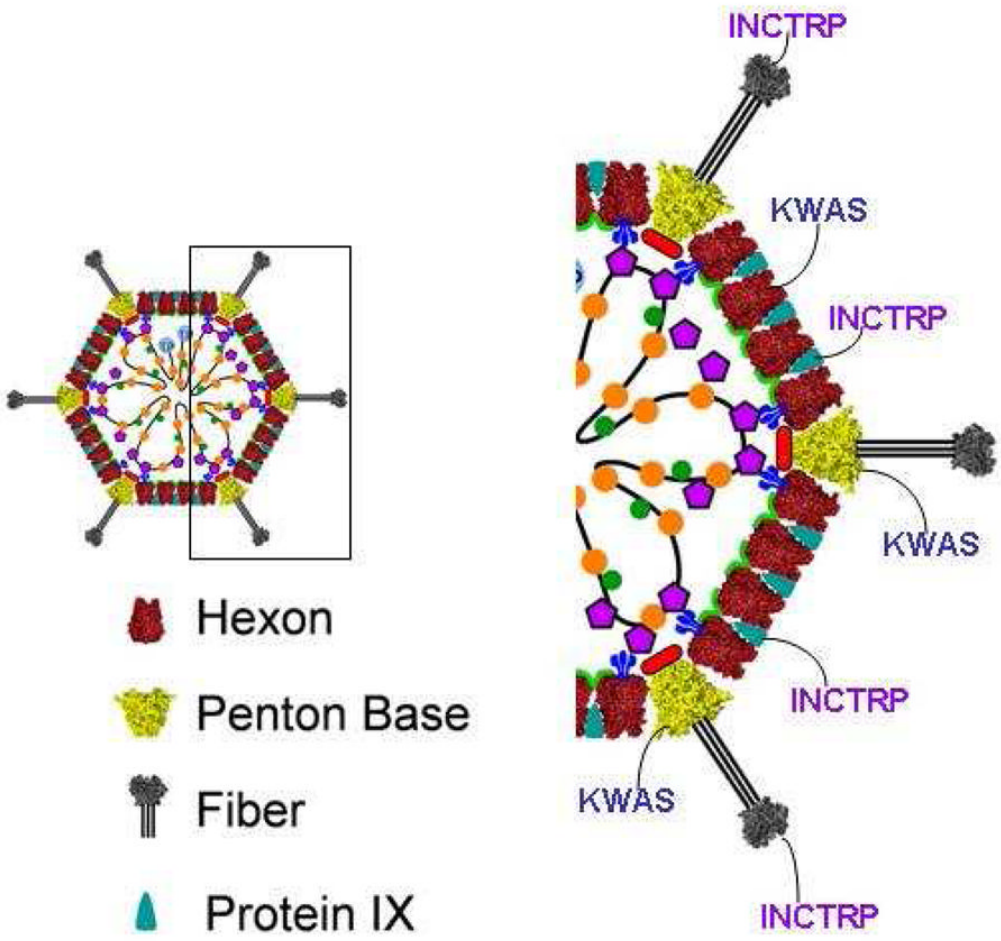


**Figure 1.**
Antigen Capsid-incorporation within Adenovirus Structural Proteins. Adenoviral capsid protein consists of: Hexon (II), Penton Base (III), Fiber (IV), and protein IX (pIX). Antigenic epitopes can be incorporated into these capsid structural proteins to induce antigen-specific immune responses. For example, this figure depicts the incorporation of HIV antigens glycoproteins 41(KWAS) and 120 (INCTRP). This figure is adapted from Nemerow et al., 2009. Virology 384 (2009) 380–388, copyright Elsevier.