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Multiple Adenovirus Serotypes Use αv Integrins for Infection†

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The nucleotide sequence encoding the penton base integrin-binding domains of several human adenoviruses was obtained by homology PCR. Each of the penton base proteins contains a conserved Arg-Gly-Asp (RGD) sequence that is predicted to lie at the apex of two extended alpha helices. The penton base RGD domain promotes efficient infection of host cells by multiple adenovirus serotypes via interaction with αv integrins, thus indicating that αv integrins play a central role in the entry of adenoviruses into host cells.

Infection of cells by human adenoviruses, a major cause of respiratory and gastrointestinal infections (2, 8), involves sequential interactions of the virus with two separate host cell receptors (12, 19). Although there are more than 40 different serotypes of human adenoviruses, the majority of studies of host cell interaction have been performed with adenovirus type 2 (Ad2) (3, 5, 7, 16). Initial attachment of Ad2 to cells is mediated by the 62-kDa fiber coat protein which binds to an as yet unidentified cell receptor (13). Adenoviruses from different subgroups apparently use different cell receptors for initial virus attachment (5).

Following attachment to cells via the fiber protein, Ad2 binds to cell surface integrins αvβ3 and αvβ5 via a pentavalent Arg-Gly-Asp (RGD) sequence that is present in the penton base coat protein (19). Several lines of evidence indicate that interaction of αv integrins with the penton base protein promotes efficient uptake of virus into cell endosomes. Function-blocking monoclonal antibodies to integrins αvβ3 and αvβ5 block virus internalization and infection. Cultured cells lacking αv integrins (M21-L) are also less susceptible to Ad2 infection than transfected M21-L4 cells expressing αv integrins (19). An Ad2 penton base mutant containing an RGE sequence instead of RGD also fails to support αv integrin-mediated cell adhesion, and viral mutants containing the RGE motif have decreased infection efficiency (1).

Although substantial knowledge of the early events of Ad2 entry into cells exists, relatively little information about whether human adenoviruses from different subgroups use αv integrins for infection has been obtained. In the present studies, we examined whether the penton base proteins of several human adenoviruses representing subgroups A, B, C, and E contain an RGD sequence and whether interaction of these viruses with αv integrins is required for efficient cell infection.

Nucleotide sequences and predicted secondary structure of the RGD domains of penton base proteins of different adenovirus serotypes. Our previous studies demonstrated that an RGD sequence in the Ad2 penton base mediates binding to αv integrins and also promotes uptake of virus into cells. To determine whether other adenovirus serotypes from different subgroups also contain a penton base RGD sequence, we used homology PCR (*Taq* polymerase; Cetus Corp.) to amplify the region of the penton base encoding the RGD domain. As shown in Fig. 1, PCR products of 504, 396, and 381 bp, respectively, were amplified from purified Ad2, Ad3, and Ad4 viral DNA. Following ligation of the PCR DNA fragments into the cloning vector, pCRII (Invitrogen Corp., San Diego, Calif.), and sequencing of the PCR insert, a computer-based alignment (GENALIGN; Intelligenetics) of the deduced amino acid sequences was performed. As shown in Fig. 2, the amino acid sequences of N-terminal and C-terminal regions of the penton base proteins of Ad2, Ad3, and Ad4 as well as Ad12 (1, 14) (subgroups C, B, E, and A [17], respectively) are very similar. In contrast, the amino acid sequence of the central region of the penton base is highly variable, although each of the virus serotypes contained a conserved RGD sequence, as had been previously noted for Ad12 (1). A true alignment of the central region is somewhat arbitrary because of the significant differences in size. The predicted secondary structure of each RGD domain is a helix-turn-helix with the RGD sequence displayed as an exposed turn at the apex of the two helices (Fig. 3). A similar structural motif in the E and F helices of sperm whale myoglobin has previously been identified (10). The helices of the different penton base proteins vary in length and contain approximately 18 to 55 amino acids, almost all of which are hydrophilic residues, suggesting that the RGD domain is exposed on the surface of the virus particle.

Role of αv integrins in adenovirus infection and biological significance. The presence of a conserved RGD sequence in the penton base proteins of multiple adenovirus serotypes suggested an involvement of cell integrins in infection. To examine this possibility, we measured adenovirus infection of a defined pair of cultured cell lines which either express (M21-L4) or lack (M21-L12) integrins αvβ3 and αvβ5 (6). As shown in Fig. 4, M21-L4 cells showed 5- to 20-fold-higher levels of infection by Ad2, Ad3, and Ad4 than M21-L12 cells. These studies indicated that αv integrins promote cell infection by several different adenovirus serotypes.

To substantiate this conclusion, further studies were next performed to determine whether virus infection could be inhibited by function-blocking monoclonal antibodies to αvβ3 (LM609) or αvβ5 (P3G2) or with soluble RGD peptides. As shown in Fig. 5, preincubation of cells with soluble RGD peptide, but not a control RGE peptide, inhibited Ad3 infection by approximately 90%. Incubation of cells with a function-blocking antibody to αvβ3 (LM609) or αvβ5 (P3G2) inhibited Ad3 infection by approximately 50%, while pretreatment of cells with a combination of these function-blocking antibodies blocked 90% of the Ad3 infection. Similar results were also obtained with Ad4 infection (data not shown). These studies demonstrate that the RGD motif present in several adenovirus

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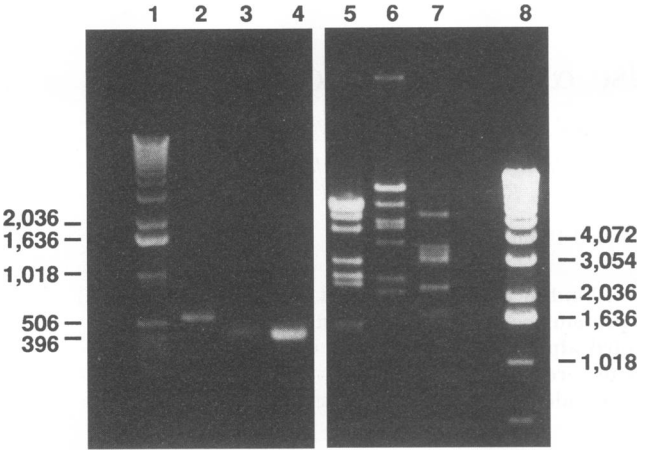

FIG. 1. Analysis of adenovirus penton base PCR products on a 1.2% agarose gel. A set of degenerate oligonucleotide primers [N terminal, 5′-TTATCTAGAATGGCCGGTCCGTGCGGIGTIGA(C/T)TT(C/T)AC-3′; C terminal, 5′-ATTGGATCCTTACGGACCGTCICCGTAGTTGTAIGC-3′] was used to amplify a region of the penton base containing the RGD sequence in Ad2 (lane 2), Ad3 (lane 3), and Ad4 (lane 4) DNA. In parallel studies, Ad2 (lane 5), Ad3 (lane 6), and Ad4 (lane 7) DNA was digested with a restriction endonuclease, *Sma*I, and the fragments were separated on a 0.8% agarose gel. The 1-kb ladder size marker was run in lanes 1 and 8.

subgroups mediates the interaction of virus with αv integrins, an event required for efficient virus infection. Previous studies by Waddell and Norrby had suggested that multiple human adenoviruses possess integrin-binding activity on the basis of their ability to cause cell rounding (18). The presence of a conserved RGD sequence in the penton base proteins of different adenovirus serotypes provides further evidence that αv integrins play a central role in virus entry. Since adenoviruses from different subgroups were reported to possess different initial receptors for attachment via the fiber coat protein (5), we sought to substantiate the use of separate fiber receptors by different adenoviruses by performing binding studies using radiolabeled virions. As shown in Fig. 6, binding

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Ad2       1   CGVDFThSRLSNLLGIRKRqPFQEGFrItYdDLEGGNIPALLDVdAYqaSlKDdteqgggg
Ad3       1   CGVDFTESRLSNLLGIRKRhPFQEGFkIMYEDLEGGNIPALLDVtAYEeSKKD........
Ad4       1   CGVDFTESRLSNmLGIRKRQPFQEGFqIMYEDLdGGNIPALLDVeAYEkSK.........e
Ad12      1   CGVDFTESRLSNiLGIRKRQPFQEGFvIMYEhLeGGNIPALLDVkkYE.............
Consensus     CGVDFTeSRLSNlLGIRKRqPFQEGF-ImYedLeGGNIPALLDV-aYe-skkddteqggg-
                                          ***
Ad2      62   aggsnssgsgAEEnsnaaaaamqpvEDmnDhaiRGDTfatraeEKraeaEAAAeaaapaaq
Ad3      54   tttettTlAVAEE.........tsED..DditRGDT...yitEK.qkrEAAA........
Ad4      53   esvaasTaAVA.............tasteVRGD....NFasaaavAaAKA........
Ad12     49   ........................nslqdqntVRGD....NF.....iAlnKA........
Consensus     -----st-avaeensnaaaaamq--ed--d--vRGDtfatnf-ek-a-aea-Aeaaapaaq
                                          ***
Ad2     123   pEVekpqKkpvIkPLteDSKkRSYNlisnDstfTqYRSWYLAYNYG
Ad3      91   aEV...kKelkIQPLEKDSKgRSYN.VLeDKiNTAYRSWYLAYNYG
Ad4      86   dE...teskivIQPVEKDSKdRSYN.VLsDKKNTAYRSWYLAYNYG
Ad12     69   .........arIePVEtDpKgRSYN.lLpDKKNTkYRSWYLAYNYG
Consensus     -evek--kk-vIqP-ekDsK-RSYNlvl-DkknTaYRSWYLAYNYG
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FIG. 2. Alignment of the adenovirus penton base sequences. Identical amino acid residues are indicated by vertical lines. Gaps indicated by dotted lines were used to maximize the alignment. The conserved RGD sequences are in bold and are indicated by asterisks. The deduced sequence for Ad12 was obtained from previously published studies (14).

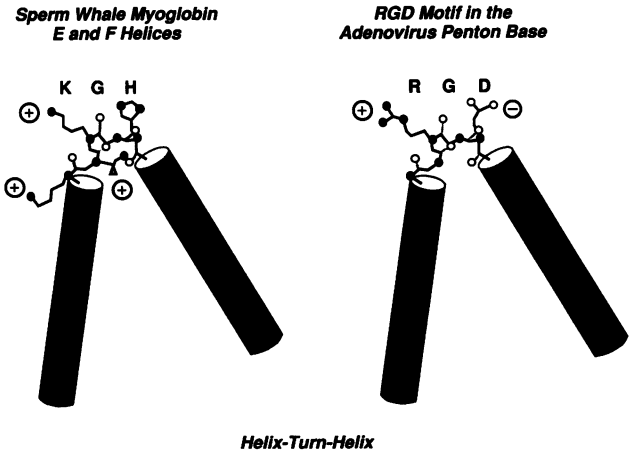

FIG. 3. The predicted secondary structure of the RGD domain of the adenovirus penton base is a helix-turn-helix. Two different structure prediction algorithms, Chou-Fasman and Garnier-Robson, were used to construct the model. A similar structural motif is present in sperm whale myoglobin (10).

of ^{3}H-labeled Ad3 to cells was blocked by a 50-fold excess of unlabeled Ad3 but not by the same amount of unlabeled Ad2. Similarly, binding of ^{3}H-labeled Ad2 was blocked by an excess of unlabeled Ad2 but not by Ad3 (not shown). These findings indicate that virus internalization receptors (αv integrins) rather than virus attachment receptors (fiber receptors) represent a common pathway for adenovirus entry into cells. It is not yet known whether any of the over 40 other adenoviruses contain penton base RGD sequences; however, Ad40, another serotype (4), does not contain this motif. The lack of an RGD

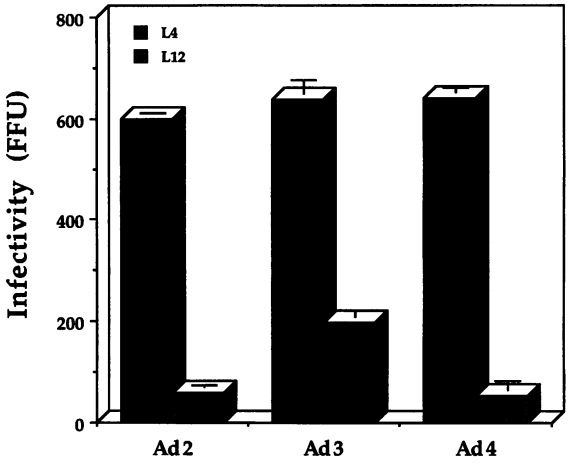

FIG. 4. Infection of M21-L4 cells expressing αv integrins (solid bars) or of M21-L12 cells lacking these receptors (shaded bars) by Ad2, Ad3, and Ad4. M21 cells (7×10^4) were incubated in solution at 4°C for 1 h with approximately 100 virus particles per cell. The cells were then warmed to 37°C for 40 min to induce internalization. Noninternalized cell-associated virions were subsequently removed by treatment with trypsin-EDTA (19) and then plated on poly-L-lysine-coated tissue culture wells. Under these conditions, approximately 1 to 10% of the cells were infected. Virus infection was quantitated by the fluorescent focus assay at 48 h postinfection. FFU, fluorescence focus units.

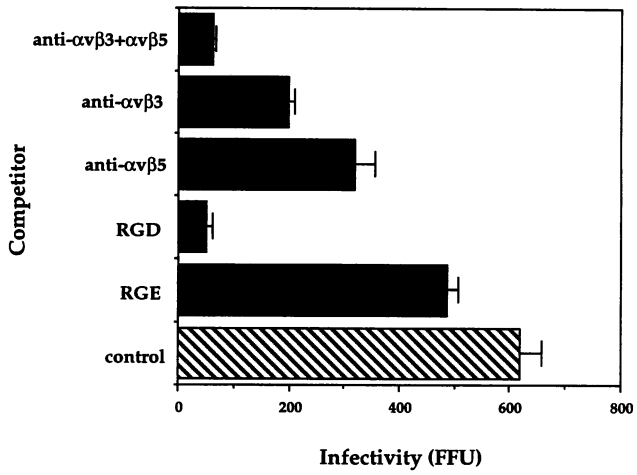

FIG. 5. Inhibition of Ad3 infection of M21-L4 cells with monoclonal antibodies to αv integrins or RGD peptides. Cells were preincubated for 60 min at 4°C with 100 μg of function-blocking antibodies to αvβ3 (LM609), function-blocking antibodies to αvβ5 (P3G2), or a combination of these antibodies per ml or with 4 mg of GRGDSP or GRGESP synthetic peptide per ml prior to the addition of 100 particles of Ad3 per cell. Virus infection was performed as described in the legend to Fig. 4 and quantitated by the fluorescent focus assay. FFU, fluorescence focus units.

sequence in Ad40 and perhaps other adenoviruses does not rule out the involvement of integrins in infection, however, since it is known that non-RGD sequences are also capable of mediating integrin binding (9).

The predicted secondary structure of the RGD domains of several adenoviruses suggests that the RGD sequence forms a tight beta turn between two extended alpha helices, suggesting that this domain is a well-defined structure. This predicted structural motif is somewhat different from the RGD integrin-binding domain present in the tenth type III module of fibronectin. Studies of the three-dimensional structure of this cell matrix protein indicate that this RGD sequence lies on an exposed loop between two beta strands and that it is also a flexible structure (11). Interestingly, image reconstructions of Ad2 obtained by cryoelectron microscopy combined with X-ray crystallography revealed a 10 Å (1 nm) protrusion on each polypeptide subunit of the penton base (15). It will be of interest to determine whether these protrusions correspond to the RGD domains, since their extension away from the surface of the penton base protein would facilitate interaction with cell surface αv integrins. Finally, the conserved RGD sequence in adenovirus penton base proteins may allow the development of specific antiviral agents capable of inhibiting the interaction of multiple adenovirus serotypes with αv integrins and, thus, capable of blocking infection.

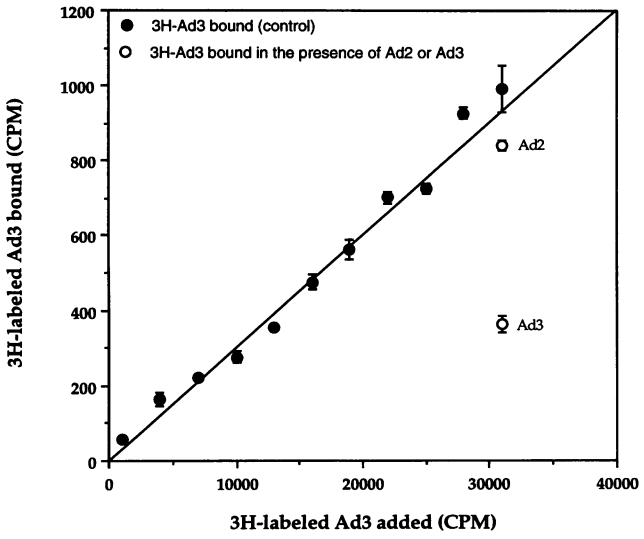

FIG. 6. Analysis of the binding of Ad3 to host cells. Various amounts of [^{3}H]thymidine-labeled Ad3 were incubated with 1×10^6 SW480 human carcinoma cells for 30 min at 4°C. In parallel studies, SW480 cells were preincubated for 60 min at 4°C with a 50-fold excess of unlabeled Ad2 or Ad3 prior to the addition of [^{3}H]thymidine-labeled Ad3. The cells were then washed three times in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered saline, pH 7.4, containing 1% bovine serum albumin and then counted. CPM, counts per minute.

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