Common epitope on protein VI of enteric adenoviruses from subgenera A and F

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Western blot analysis with monoclonal antibodies, produced in response to immunization with gradient-purified adenovirus 41 (Ad41) virions, identified two epitopes of interest on protein VI of enteric adenoviruses. One epitope is unique to subgenus F adenoviruses (Ad40 and Ad41); the other epitope is common to subgenus A (Ad12, 18 and 31) and subgenus F (Ad40, 41) adenoviruses but is not shared by representative serotypes of subgenera B (Ad3 and 7), C (Ad1, 2 and 5), D (Ad8) or E (Ad4). Alignment of the deduced amino acid sequence of the genes encoding the protein VI precursor (pre-VI) of Ad40 and Ad41 (subgenus F), Ad12 and Ad31 (subgenus A), Ad2 and Ad5 (subgenus C) shows that the N-terminal one-third and C-terminal 23 amino acids of pre-VI are highly conserved. Within the central domain, pre-VI of subgenus F serotypes is more closely related to that of subgenus A serotypes than to pre-VI of the non-enteric subgenus C adenoviruses (Ad2 and Ad5). By expressing random oligonucleotide fragments of the Ad41 protein VI gene as part of a T7 gene 10 fusion protein, the two epitopes of interest were mapped to within the same 14 amino acid region in the central domain of protein VI. Given the association of subgenera A and F adenoviruses with paediatric gastroenteritis, the epitope shared by these serotypes may be functionally significant with respect to gut tropism. In addition, this epitope is potentially valuable as a target for the detection of enteric adenoviruses in clinical specimens.

Introduction

Adenoviruses are now recognized as important agents of paediatric gastroenteritis. Serotypes most frequently associated with gastroenteritis include those of subgenus A and subgenus F (Brown et al., 1984; Brown, 1990; de Jong et al., 1993; Johansson et al., 1994; Mickan & Kok, 1994; Noel et al., 1994; Grimwood et al., 1995; Scott-Taylor & Hammond, 1995). Other serotypes (subgenera B, C, D and E) are rarely associated with gastroenteritis (Brown, 1990) even though some of these serotypes (e.g. those of subgenus C) have the ability to replicate in a variety of tissues. Subgenus A and F serotypes can therefore be referred to as 'enteric' adenoviruses based on their shared tropism for the gastrointestinal (GI) tract in the natural host. However, subgenus F adenoviruses exhibit a more restricted tropism than subgenus A serotypes. Whereas Ad31 can cause disseminated disease in immunocompromised hosts (Rodriguez et al., 1984; Johansson et al., 1991; Hierholzer, 1992; Schnurr et al., 1995), there is no evidence that Ad40 and Ad41 spread to cause disease outside the GI tract even in immunocompromised individuals (Schofield et al., 1994; M. Brown, unpublished results). In cell culture, Ad40 and Ad41 grow poorly (relative to other serotypes) and are thus referred to as 'fastidious' enteric adenoviruses (FEAds). This poor replicative potential of the FEAds in cell culture can be attributed, at least in part, to the cumulative effect of partial blocks at multiple stages in the replicative cycle. These blocks, which presumably reflect the absence of specific host cell factors, include low-level expression of the E1B 55K protein (Mautner et al., 1989; Bailey et al., 1993, 1994) as well as inefficient encapsidation of the genome (Tiemessen & Kidd, 1994; M. Brown, unpublished results) coupled with inefficient release of progeny virus (Brown et al., 1992). The fastidious growth characteristics of subgenus F adenoviruses have
recently been reviewed in detail by Mautner et al. (1995) and by Tiemessen & Kidd (1995).

The MAbs which form the basis of the present study were prepared to facilitate characterization of the FEAds. These MAbs have provided information which may be relevant to understanding protein interactions within the virion and are potentially valuable for the identification of enteric adenoviruses in clinical specimens.

**Methods**

**Virus and cells.** Prototype strains of Ad4, Ad12, Ad18, Ad40 (Dugan) and Ad41 (Tak) were obtained from the American Type Culture Collection (ATCC). The prototype strain of Ad3 was obtained from Dr N. Singh-Naz, Children's Hospital, National Medical Center, Washington, DC; Ad5 was obtained from Dr F. Graham, McMaster University, Hamilton, Ontario, Canada. Ad8 was isolated from conjunctival specimens from patients with conjunctivitis at the Wellesley Hospital, Toronto, Canada. Ad31 was isolated from stool specimens from patients with gastroenteritis at the Hospital for Sick Children, Toronto, Canada. Isolates were serotyped by microneutralization tests using specific antisera from the ATCC. All serotypes were propagated in 293 cells, a continuous line of transformed human embryonic kidney cells (Graham et al., 1977) as described previously (Brown et al., 1984). The 293 cell line was also acquired from Dr F. Graham.

Hybridoma cells producing the MAbs of interest were prepared by standard techniques (Harlow & Lane, 1988) using NS-1 myeloma cells fused with spleen cells from BALB/c mice that had been immunized with gradient-purified Ad41 virions, as described previously (Fortsas et al., 1994). Hybridoma cells were then propagated as described previously (Fortsas et al., 1994).

transferred to nitrocellulose and screened with one of the two MAbs for expression of the corresponding epitope. The protocol used for colony transfer and immunoscreening was that supplied with the pTope T-Vector kit (Novagen). For confirmation of epitope expression by Western blot analysis, positive colonies were picked and grown overnight in 2YT broth. The cells were collected by centrifugation (5000 g for 2 min) then resuspended in sample buffer [50 mM-Tris–HCl pH 6.8, 1% SDS (w/v), 0.14 M-2-mercaptoethanol, 10% glycerol (v/v) and phenol red] and boiled for 5 min prior to SDS–PAGE. Colonies expressing the epitopes of interest were then amplified for extraction of plasmid DNA for sequence analysis. The epitopes were mapped within the protein by aligning the expressed peptide sequences with the entire protein VI sequence.

Results

Antigen/serotype specificity of MAbs

Hybridoma supernatants were initially screened by immunofluorescent staining of 293 cells infected with Ad40 (Dugan), Ad41 (Tak) and Ad1. MAbs specific for Ad40 and Ad41 were then used to screen cells infected with viruses from 36 stool specimens, collected over the period from May 1987 to December 1988, from children with diarrhoea at The Hospital for Sick Children, Toronto. The serotype contained within each specimen was determined by SmaI restriction analysis of viral DNA extracted from infected 293 cells as previously described (Brown, 1990). All 36 cultures were positive by immunofluorescence microscopy using control MAb GS which recognizes a genus-specific epitope on hexon trimers and higher-order structures (Fortasas et al., 1994). Two other MAbs each gave positive results with 8 Ad40-infected cultures and 25 Ad41-infected cultures but not with cultures infected with Ad2 (subgenus C), Ad3 or Ad7 (both subgenus B) (results not shown).

The serotype specificity of these two MAbs was further examined, by immunoblot analysis, with subgenus A serotypes (Ad12, 18 and 31) as well as representative serotypes of subgenera B (Ad3), C (Ad5), D (Ad8) and E (Ad4), with Ad40 and Ad41 as positive controls. Infected cell lysate was used as the source of viral proteins for this experiment and a duplicate blot was incubated with anti-hexon MAb GS (Fortasas et al., 1994) to confirm that adequate amounts of adenovirus protein were present on the blot. High molecular mass bands, characteristic of non-denatured hexon species, were detected in lysates of cells infected with all serotypes tested (Fig. 1a). In contrast, one of the MAbs of interest (K) detected a complex of bands in lysates of cells infected with all subgenus A serotypes (Ad12, 18 and 31) and subgenus F serotypes (Ad40 and 41) but not in lysates of cells infected with serotypes of subgenus B, C, D or E (Fig. 1b). The other MAb of interest (B) detected the same complex of bands only in lysates of cells infected with Ad40 and Ad41 (results not shown).

The two MAbs of interest were characterized (with respect to antigen specificity) by immunoblot analysis using virion proteins from caesium chloride gradient-purified Ad40, Ad41 and Ad5 virions. Both MAbs reacted with a protein migrating in the position of protein VI; the results for MAb K are shown in Fig. 2. The single band corresponding to protein VI in Fig. 2 represents the mature protein. The additional protein VI bands seen in Fig. 1 (b) represent the protein VI precursor (pre-VI) and the cleavage intermediate (iVI) which are present in cell lysate.

DNA sequence analysis

In order to define the epitopes of interest, the nucleotide sequences of the pre-VI genes of Ad31, Ad40 and Ad41 were determined for comparison of the deduced amino acid (aa) sequence with those of Ad12, Ad2 and Ad5 (Freimuth et al., 1993; Sprengel et al., 1994; Roberts et al., 1986; Chroboczek et al., 1992). The nucleotide sequence of the Ad40 pre-VI gene determined in this laboratory is identical to the sequence which was published by Davison (1993) after this work was initiated.
Fig. 2. Antigen specificity of MAb K. Proteins of 35S-labelled, gradient-purified virions were separated by SDS-PAGE and transferred to nitrocellulose membranes for reaction with MAb K. Samples were not boiled prior to electrophoresis. Total protein profiles were visualized by autoradiography of the membranes. Ad5 proteins are indicated on the right.

Aligned sequences are shown in Fig. 3 with identical residues shaded. The corresponding sequences of three non-human adenoviruses are shown for comparison: murine adenovirus type 1 (MAd1) (Song et al., 1995), ovine adenovirus strain 287 (OAV) and fowl adenovirus type 1 (FAV1) (CELO). The partial sequence for protein VI of an avian adenovirus associated with haemorrhagic enteritis (HEV type A) is also shown.

The length of the pre-VI sequence in human adenoviruses ranges from 250 amino acids (aa) in Ad2 and Ad5 to 267 aa in Ad40. Enteric adenoviruses 12, 31 and 41 have sequences (260–266 aa) which are slightly shorter than that of Ad40. The non-human adenoviruses have sequences (221–237 aa) which are shorter than those of human adenoviruses.

The amino-terminal 100 aa and the carboxy-terminal 23 aa are highly conserved among the six human adenovirus (HAd) serotypes. Fig. 4 summarizes the relationship between pre-VI of different HAd serotypes in each of the conserved terminal domains and in the divergent central domain. Values for each pair of serotypes represent the number of identical and conserved residues expressed as a percentage of the total number of residues in the longer of the two proteins. Within the central domain, proteins from serotypes of the same subgenus share at least 85% identical amino acids and more than 90% conserved amino acids. In the same region, subgenus F (Ad40 and 41) sequences are more closely related to subgenus A (Ad12 and 31) sequences than to subgenus C (Ad2 and 5) sequences, with 43–47% identical and 61–64% conserved amino acids between subgenera F and A but only 33–36% identical and 41–47% conserved amino acids between subgenera F and C. Overall, 54% of the residues in Ad40 pre-VI are identical among all six HAd serotypes and 69% of the residues are conserved. An additional 13% of the residues are conserved among enteric adenoviruses (subgenera F and A) but not between enteric and non-enteric adenoviruses (subgenus C); these residues are designated with an asterisk in Fig. 3.

There is little conservation between the MAd1 sequence and that of the HAd serotypes except in the terminal domains (Fig. 3). Identical amino acids shared by MAd1 and the six HAd serotypes account for 51% and 61% of the residues in the amino- and carboxy-terminal domains, respectively, but only 8% of the residues in the central domain. There is little evidence of conservation between MAd1 and the other non-human adenovirus sequences. Based on the alignment shown in Fig. 3, OAV strain 287 shares 33% identical amino acids overall with avian adenovirus FAV1 but only 17% identical amino acids with mastadenovirus MAd1. However, the consensus sequences for the two protease cleavage sites [(M,L,I)XGG-X and (M,L,I)XGX-G] (Webster et al., 1989) are conserved among the human and non-human adenoviruses (Fig. 3).

Epitope mapping

A total of 11 transformants were selected: 7 by screening with MAb K (specific for subgenera A and F) and 4 by screening with MAb B (specific for subgenus F). A representative immunoblot of cell lysate, used to confirm expression of the epitopes, is shown in Fig. 5. The four transformants represented in lanes 3–6 all express the epitope recognized by MAb B (unique to subgenus F) whereas only three of them express the epitope recognized by MAb K (common to subgenera A and F). Transformant B2 (Fig. 5, lane 5) was the only one of 11 transformants which expressed a single epitope; the other transformants expressed both epitopes (Fig. 6). Based on alignment of the inserted amino acid...
Fig. 3. Amino acid alignment of pre-V1 protein. Corresponding DNA sequences were obtained in this laboratory for Ad40, Ad41 and Ad31 and from published data for Ad2 (Roberts et al., 1986), Ad5 (Chroboczek et al., 1992), Ad12 (Freimuth & Anderson, 1993). GenBank accession numbers are as follows: Ad2, J01917; Ad5, M73260; Ad12, L02237 and X73487; Ad31, U14653; Ad40, U14651; Ad41, U14652; MAdl, X74742; OAV strain 287, U40837; FAV1 (CELO), Z67970; HEV type A, U31805. Sequences were aligned using DNASIS for Windows Version 2.0 with subsequent adjustment by eye. Identical amino acids among five or more serotypes are shaded. Amino acids which are conserved among the enteric HAd serotypes (Ad12, 31, 40, 41) but not between the enteric and non-enteric HAd serotypes (Ad2, 5) are indicated with an asterisk (*). Residues are considered to be conserved if exchanged within the same group of amino acids as follows: (i) small (A, P, S, T); (ii) hydrophobic (I, L, M, V); (iii) basic hydrophilic (K, R); (iv) neutral hydrophilic (N, Q); (v) acidic hydrophilic (D, E); (vi) aromatic (F, W, Y). Residues C, G and H were treated as unconserved. The peptide encompassing epitopes B and K is shown in bold type. Vertical arrows mark the cleavage sites used by the adenovirus protease.
Amino terminus (aa 1–100)

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Carboxy terminus (aa 244–267 of Ad40)

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sequences expressed by the 11 transformants (Fig. 6), both epitopes map within the sequence EEKLPPLETAPPLP in the central domain of protein VI (Fig. 3). The fact that clone B2 expresses epitope B but not K indicates that the first glutamic acid residue in this peptide constitutes part of the epitope common to subgenera A and F adenoviruses.

**Discussion**

Protein VI is an internal virion protein, located beneath the vertices, connecting the ring of peripental hexons from within the interior of the capsid (Stewart et al., 1993). Protein VI also has DNA-binding properties (Russell & Precious, 1982) and may be important in connecting the capsid to the core (Stewart et al., 1993). It is one of several virion proteins (IIIa, VI, VII, VIII, μ and terminal protein) which are synthesized as precursor molecules and then cleaved by the virion protease as the final step in virion maturation. In fact, the C-terminal 11 aa peptide of pre-VI has been shown to be an important co-factor for protease activity (Mangel et al., 1993; Webster et al., 1993; Keefe et al., 1995).

Cleavage occurs by way of a single intermediate, represented by the middle of three bands, clearly evident in Fig. 1 (b). In this particular figure, the three bands are most easily seen in the lane loaded with Ad18-infected cell lysate. The relative intensity of the three bands, which presumably represent the complete precursor, the cleavage intermediate and the mature protein, is variable in different preparations of cell lysate, even when the same virus inoculum is used to infect the cells (results not shown). A cleavage intermediate has also been documented in processing of pre-VI to VI in Ad2- and Ad4-infected cells (Matthews & Russell, 1994) and thus is not unique to enteric adenoviruses. The intermediate, vi, appears to be derived from pre-VI by removal of the C-terminal 11 aa peptide (Matthews & Russell, 1994, 1995).

The deduced amino acid sequences of six human adenoviruses representing three subgenera (A, C and F) show a high degree of conservation in both the N-terminal and C-terminal domains of pre-VI (Figs 3, 4). These domains not only encompass the cleavage sites but contain sequences (corresponding to aa 48–74 and 233–239 of Ad2 pre-VI) near the termini of the mature protein, which are important for direct interaction with hexon trimers (Matthews & Russell, 1994, 1995). The cleavage sites are conserved among mastadenoviruses, including Mad1 and OAd1, and in avianadenoviruses AAd1 and HEV. The hexon-binding sequences, however, are not conserved in OAd1 or in the avianadenoviruses.

The two epitopes identified in this study both map within a 14 aa sequence (EEKLPPLETAPPLP) in the central domain of protein VI. Epitope K, shared by subgenera A and F adenoviruses, is likely to be contained within the sequence EEKLPLL, common to these four serotypes (Fig. 3). The first glutamic acid residue in this sequence is part of the tripeptide VEE which is conserved among the six HAd serotypes examined and is crucial for recognition by MAb K (Fig. 6). Epitope B, unique to subgenus F adenoviruses, is likely to be defined, at least in part, by the sequence unique to Ad40 and Ad41 i.e. APPL (Fig. 6). In keeping with the internal location of these epitopes within the capsid, neither of the corresponding MAb neutralized virus infectivity nor did they bind to virois as determined by immunogold-labelling experiments (results not shown).

The initial observation that MAb K recognized protein VI of serotypes within subgenera A and F, but not other subgenera, was intriguing given the association of both subgenera A and F serotypes with gastroenteritis (Brown, 1990). It is now recognized that the presence of a common
epitope on protein VI of subgenera A and F adenoviruses is in keeping with the close phylogenetic relationship of these serotypes, as shown by comparison of nucleotide sequence data for the ITR, E1, E2a, E3b, major late promoter, hexon, protease and fibre regions of the genome (Bailey & Mautner, 1994). An interesting possibility is that the epitope has a functional significance with respect to tropism.

Epitope K represents a potentially valuable target in screening specimens for clinically relevant adenoviruses, from subgenera A and F, using a single test. Existing immunoassays, which employ MAbs directed against type-specific epitopes on Ad40 or Ad41 hexon, detect only subgenus F adenoviruses (Herrmann et al., 1987; van der Avoort et al., 1989; Wood et al., 1989; Cruz et al., 1990; Lew et al., 1991; Van et al., 1992; de Jong et al., 1993; Jarecki-Khan et al., 1995; Noel et al., 1994; Ahluwalia et al., 1995; Grimwood et al., 1995). Furthermore, the emergence of hexon variants could compromise the value of existing immunoassays. Several Ad41 variants have been found to escape detection by particular anti-hexon MAbs (van der Avoort et al., 1989; Scott-Taylor et al., 1990; Noel et al., 1994) and pooled MAbs were found to decrease, rather than increase, the sensitivity of the assay (Noel et al., 1994). The MAbs identified in the present study have the potential to detect multiple variants of Ad41 expressing different hexon epitopes. When used for immunofluorescent staining of infected cells, both MAbs B and K identified 11 specimens containing Ad41 strains which escaped detection by an early version of the Adenoclone (40/41) kit (Cambridge Bioscience) (results not shown). The modified kit now detects the Ad41 strains presently circulating in North America (Scott-Taylor et al., 1990; Van et al., 1992; Ahluwalia et al., 1995) but the emergence of new variants remains a potential problem. Internal epitopes B and K thus represent alternative targets for immunoassay which are likely to be more stable than those epitopes expressed on the surface of the virion and hence subject to antibody-mediated selection.

The technical assistance of Sumita Fleming in preparation of the MAbs is greatly appreciated. The authors thank Prof Frances Doane, Department of Microbiology, University of Toronto, for testing the monoclonal antibodies in immunogold-labelling experiments. Development of the MAbs was supported by funds from The Hospital for Sick Children Research and Development Limited Partnership. The remaining work was supported by grant MA11333 from the Medical Research Council of Canada.

References


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Epitope K
Epitope B

Fig. 6. Alignment of peptides expressing epitopes B and K. Clones K1–K7 were selected by immunoscreening with MAb K; clones B1–B5 were selected by immunoscreening with MAb B.


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