Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics

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Two canine isolates of simian virus 5 (SV5), termed CPI+ and CPI−, were examined for their ability to react with a bank of monoclonal antibodies (MAbs) that had been previously raised against a human isolate of SV5. CPI− virus was originally isolated from the brain of a gnotobiotic dog infected with CPI+ virus and establishes persistent infections more readily than CPI+ in vitro. Of more than 50 MAbs tested, only one (P-k) reacted with CPI+ but not CPI−, enabling distinction between the two canine isolates. It had been shown previously that MAb P-k reacts with an epitope common to both the P and V proteins. In order to characterize further the epitope binding site of this MAb the P/V genes of CPI+ and CPI− were sequenced. There were four nucleotide differences between CPI+ and CPI−, three of which resulted in predicted amino acid substitutions. Synthetic peptides corresponding to regions encompassing these changes were made and radioimmune competition assays were used to identify the epitope binding site of MAb P-k. Sequence comparison of the P/V gene of CPI+ with the published sequence of a monkey isolate of SV5 (W3) revealed 14 nucleotide differences with five amino acid substitutions. The only amino acid substitution observed between CPI+, CPI− and W3 which altered the predicted secondary structures of the P and V proteins was a leucine to proline change that induced a predicted β-turn and resulted in the loss of binding of MAb P-k.

Introduction

Simian virus 5 (SV5) was originally isolated from rhesus monkey kidney cell cultures (Hull et al., 1956) but has subsequently been isolated from tissues of other animals including humans (Hsiung, 1972; Robbins et al., 1981; Goswami et al., 1984) and dogs (Rosenberg et al., 1971; McCandlish et al., 1978). SV5 causes tracheo-bronchitis in dogs and is often associated with kennel cough (McCandlish et al., 1978). Thus in veterinary circles SV5 is usually referred to as canine parainfluenza virus (CPI). SV5 is a member of the paramyxovirus family, being most closely related to mumps virus and parainfluenza virus type 2 (Ito et al., 1987; Randall & Young, 1988; Precious et al., 1990; Southern et al., 1990; Kawano et al., 1990). The negative-sense single-stranded RNA genome encodes, from the 3’ end, eight virus proteins termed the nucleo- (NP), phospho- (P), V, matrix (M), fusion (F), small hydrophobic (SH), haemagglutinin-neuraminidase (HN) and large (L) proteins. All the virus proteins, except for SH, have been detected in purified virions. The HN and F proteins are glycosylated and form spikes that protrude through the virus envelope. The M protein is located on the inner surface of the virion envelope and the NP, P, L and possibly V proteins associate with genomic RNA to form a ribonucleoprotein complex termed the nucleocapsid (McSharry et al., 1975; Buetti & Choppin, 1977). By analogy with other paramyxoviruses it is likely that the NP, P, L and probably V proteins all play a role in virus transcription and replication. The P and V proteins are encoded by the same gene. As with other paramyxoviruses, the mechanism by which the P/V gene encodes both proteins is by the insertion of non-templated G residues at precise positions during mRNA synthesis (Thomas et al., 1988; Southern et al., 1990). We have previously reported the isolation and characterization of a bank of monoclonal antibodies (MAbs) to the HN, F, M, NP and P and V proteins of SV5 and we used these MAbs to show that there were only minor antigenic differences between human, simian and canine isolates of the virus (Randall et al., 1987).

Like other members of the paramyxovirus family SV5 can establish persistent infections in vitro and in vivo. Indeed it has been suggested that the common contamination of primary monkey cell lines with SV5 occurs because monkeys may be persistently infected with the virus (Hsiung, 1972). SV5 can also establish a prolonged or persistent infection in immunodeficient mice and we have used this experimental model system to analyse the relative importance of different immune responses in...
clearing persistent paramyxovirus infections. In dogs it appears that SV5 may also establish persistent infections. A neurotropic strain of SV5, termed CPI+, was isolated from the cerebrospinal fluid of a dog with temporary posterior paralysis (Evermann et al., 1981; Baumgartner et al., 1981). Intracerebral infection of gnotobiotic dogs with CPI+ resulted in acute encephalitis with laminar cortical necrosis and the presence of viral antigens was demonstrated in ependymal cells and neurons (Baumgartner et al., 1982). During the course of these studies virus was re-isolated from infected brain tissues. The recovered isolate, termed CPI−, differed in its c.p.e. in tissue culture from that of the parental CPI+ virus. Interestingly, CPI− established persistent infections in tissue culture cells more readily than CPI+ (Baumgartner et al., 1987). Here we show that of more than 50 MAbs to SV5, only one, with specificity to the P/V protein, distinguished between these two virus isolates. The epitope binding site of this MAb has been finely mapped to a region common to the P and V proteins.

**Methods**

**Cells and viruses.** Vero cells (Flow Laboratories) were grown as monolayers in 75 cm² tissue culture flasks, 150 mm² Petri dishes and in rotating 80 oz Winchester bottles in Dulbecco's modification of Eagle's MEM supplemented with 10% newborn calf serum. The strain of SV5 designated W3 was isolated from a primary culture of rhesus monkey kidney cells (Choppin, 1964). W3 and the canine isolates CPI+ and CPI− (Baumgaertner et al., 1982) were grown and titrated under appropriate conditions in Vero cells using medium containing 2% calf serum (Randall et al., 1987).

Preparation of total cell RNA, cDNA synthesis, polymerase chain reaction (PCR) amplification and cloning. Vero cells, grown in 150 mm² Petri dishes, were infected with CPI+ or CPI− virus at an m.o.i. of 0.1 p.f.u. for 1 h at 37 °C. The inoculum was removed and the cells were incubated at 37 °C for 24 h in the presence of actinomycin D (5 µg/ml; Paterson et al., 1984) in MEM without calf serum. Cells were harvested and the RNA was extracted from cytoplasmic extracts as described by Gough (1988). cDNA synthesis and PCR amplification were based on the methods described by Kawasaki (1990). The PCR products were end-repaired and blunt-end ligated into M13mp8 and mp19 (Scharf, 1990). Sequencing was carried out using the method of Sanger et al. (1977). Computer-assisted alignments and comparison of sequences with other related virus genes was carried out using the Staden programs (Staden, 1982a, b) on a DEC VAX computer.

**Oligonucleotide synthesis and PCR.** Oligonucleotide synthesis was carried out using an Applied Biosystems oligonucleotide synthesizer and phosphoramidite chemistry. Oligonucleotides were used in the PCR as described in Results.

**Peptide synthesis.** Peptides were synthesized by solid phase fluorenyl methoxycarbonyl polyamide chemistry using CRB Pepsynthesiser II (Atherton et al., 1988), and purified by reversed phase FPLC (Webster et al., 1989).

**Radioimmune peptide competition assay.** MAbs were titrated against infected cell antigens bound to nitrocellulose using an assay that has been described in detail elsewhere (Randall et al., 1984). A modification was incorporated in that the nitrocellulose sheet was sandwiched between 84-well Terasaki plates (10 µl of diluted antibody per well). Bound antibody was detected with 125I-labelled Protein A (Amersham). Peptides were tested for their ability to compete with the binding of antibodies by making twofold dilutions of the peptides (starting at a concentration of 10 µg/ml, 10 µl per well) in an appropriate concentration of antibody in PBS containing 1% BSA. The antibodies were then reacted with virus antigen bound to nitrocellulose as described.

**Secondary structure analysis.** Secondary structure prediction studies on the P proteins of the W3, CPI+ and CPI− strains of SV5 were performed using the analyses of both Chou & Fasman (1978) and Garnier et al. (1978), with the software of Devereux et al. (1984) on the University of Wisconsin sequence analysis package.

**Western blotting.** Infected cell monolayers were lysed in SDS-PAGE disruption buffer (0.05 M-Tris-HCl pH 7.0, 0.2% SDS, 5% 2-mercaptoethanol and 5% glycerol), sonicated using an MSE ultrasonic probe, and heated for 5 min at 100 °C and the dissociated polypeptides separated by electrophoresis through a 12% SDS-polyacrylamide slab gel. The separated polypeptides were transferred to nitrocellulose using a semi-dry gel electrobetter. The nitrocellulose was then reacted with MAbs to the P/V protein and bound antibody was detected by 125I-labelled protein A and autoradiography (Randall & Young, 1988).

**Results**

**Antigenic analysis of CPI+ and CPI−**

Fifty-three MAbs (raised against the human LN isolate of SV5; Randall et al., 1987) were tested, in radioimmunoassays, for their ability to bind to cells infected with CPI+, CPI− and W3. The specificity of the MAbs and a summary of the results are given in Table 1. Of the MAbs tested, HN-4b, HN-x and F-1a did not react with either CPI+ or CPI−. The MAb HN-x failed to react with cells infected with W3, CPI+ or CPI−. MAb P-k was the only antibody that distinguished between cells infected with CPI+ and CPI−. It had been previously shown that this MAb reacted with an epitope common to both the P and V proteins (group 1 MAb, Thomas et al., 1988). Thus, although MAb P-k binds to both the P and V proteins of CPI+ in Western blots it fails to react with either of the CPI− proteins (Fig. 1).

**Sequence analysis of the P/V genes of CPI+ and CPI−**

In order to facilitate the rapid sequencing of the P/V genes of the CPI+ and CPI− canine isolates of SV5, we amplified cDNA made from total cell RNA by PCR and cloned the amplified products directly into M13 vectors for sequencing. From the published sequence of the P/V gene of the W3 virus isolate (Thomas et al., 1988), a set of six oligonucleotide primers were synthesized which hybridized to different regions of the P/V gene. Primers I
Table 1. Summary of specificities and reactivities of SV5 MAbs with the W3, CPI+ and CPI− virus isolates

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* MAb nomenclature is as described in Randall et al. (1987). Each MAb has an upper case letter corresponding to the virus protein recognized, followed by a trivial lower case letter as identifying labels for individual antibodies. The MAbs to the HN protein were also placed in five groups, each of which was given a group number depending on their ability to compete with one another. MAbs HN-x, -y and -z were not grouped.

Fig. 1. Autoradiogram of Western blot demonstrating the reactivity of MAbs P-k (panel a) and P-e (panel b) with cell extracts of cells infected with CPI+ (lane 1, panel a) or CPI− (lane 2, panel a; panel b). MAb P-k has been shown to react with an epitope at the N terminal end of both the P and V proteins, and P-e reacts with a C-terminal P-specific epitope (group I and group III antibodies respectively; Thomas et al., 1988). Thus the minor bands in panel (b) are P-related products. Infected cell extracts were separated by electrophoresis through a 12% polyacrylamide gel prior to Western blotting.

Fig. 2 shows the derived nucleotide sequences of the P/V gene of CPI+ and CPI− compared to the published sequence for W3. (Thomas et al., 1988). There were 14 nucleotide differences between the CPI+ and W3 isolates and a further four nucleotide differences between CPI+ and CPI−. All but one of the 18 nucleotide substitutions were either C to T or G to A transition substitutions.
There were five predicted amino acid substitutions between the W3 and CPI+ isolates and an additional three amino acid substitutions between CPI+ and CPI− viruses (Fig. 3). In no case were any amino acid substitutions observed in V-specific sequences, corresponding to the region of the genome shown to be the most conserved amongst different paramyxoviruses (Thomas et al., 1988; Southern et al., 1990).

Reaction of MAb P-k with synthetic peptides
Since MAb P-k reacted strongly with both the P and V proteins of CPI+ in Western blots (Fig. 1) its ability to
Epitope on the P and V proteins of SV5

1555

1 2

w3 MDPTDLFSIPDEINKLJETGLNTVEYFTSQQTVGYTSSLGLKNTIPPPQGVTLGLTNAAE
CPI+ H I P 1
CPI- I

Fig. 3. Differences in the predicted amino acid sequences of the P proteins of CPI+ and CPI− compared to that of the W3 isolate of SV5. The amino acids underlined denote three synthetic peptides, corresponding to regions of difference between CPI+ and CPI−, which were used in radioimmune competition assays to map the binding site of MAb P-k (Fig. 4). The amino acid sequence before the arrow is common to both the P and V proteins, after the arrow the sequence is P-specific. No amino acid differences between W3, CPI+ and CPI− were observed in V-specific sequences.

interact with appropriate synthetic peptides was examined. Three synthetic peptides were made that covered the regions in which the amino acid differences were noted between CPI+ and CPI− (Fig. 3). Radioimmune competition assays clearly demonstrated that only peptide 3, with the sequence IPNPLLGLD, competed for binding of the MAb to native protein (Fig. 4). These results clearly demonstrate that the substitution of the leucine with a proline in CPI− (Fig. 3) results in the loss of binding of MAb P-k to the native P and V proteins and that the epitope recognized by MAb P-k is located within these nine amino acids.

Discussion

Here, and elsewhere (Randall et al., 1987) we have shown that there are few antigenic differences between simian, canine and human isolates of SV5. Comparison of the P/V gene of the canine CPI+ isolate with the published sequence of the W3 simian isolate revealed about 1% nucleotide and amino acid differences between the two isolates. This is in contrast to the approximately 40% amino acid homology which SV5 has with mumps and parainfluenza virus type 2 (Precious et al., 1990; Southern et al., 1990; Kawano et al., 1990). The observation that there is little evolutionary divergence between simian and canine isolates of SV5 supports the contention that the differences observed may not prevent virus transmission between different species. Indeed it has been suggested that monkeys become infected with SV5 following their contact with man in captivity (Tribe, 1966). Similarly, although there is no good evidence that SV5 causes acute respiratory disease in man, there may be transmission of SV5 between dogs and man.

SV5, like other paramyxoviruses, is able to establish persistent infections in vitro and in vivo. Most of the evidence collected to date suggests that the establishment of persistent infections is mediated through non-specific mechanisms that involve the generation of defective interfering particles or temperature-sensitive mutants (Randall & Russell, 1991). However, in the case of CPI+ and CPI− the observation has been made that CPI− establishes persistent infections more readily in vitro than CPI+ (Baumagärten et al., 1987). Since CPI− was isolated from the brain of a gnotobiotic dog infected with CPI+ it is possible that this property was selected for in vivo. If this is the case then it is intriguing that the only antigenic difference observed between CPI+ and CPI− was in the P and V proteins. We have mapped this epitope to nine amino acid residues common to the P and V proteins and have shown that in the native protein the substitution of a leucine to proline residue in this sequence prevents MAb P-k binding to the native protein. However, these results do not exclude the

Fig. 4. Radioimmune competition assay in which three synthetic peptides (NTVEYFTSQ, GVTGLLTNAAN and IPNPLLGLD; peptides 1 to 3 in Fig. 3) were used to compete for the binding of MAbs P-k (panel a) and P-e (panel b) with cell extracts of CPI+ bound to sheets of nitrocellulose. Doubling dilutions (starting at the second well in each row) of peptides (initial concentration 10 μg/ml) were mixed with an appropriate dilution of antibody for 30 min prior to reacting the antibody–peptide mixture with the nitrocellulose. As a control, no peptide was added to the first well of each row.
This corresponds to the epitope bound by the MAb P-k. The predicted arrows indicate the area in which changes in structure are predicted for the P proteins of CPI + (structure a) and CPI− (structure b). Arrows correspond to the epitope bound by the MAb P-k. The predicted structure for W3 was identical to that of CPI+.

Fig. 5. Garnier-Osguthorpe-Robson secondary structure predictions for the P proteins of CPI + (structure a) and CPI− (structure b). Arrows indicate the area in which changes in structure are predicted. This corresponds to the epitope bound by the MAb P-k. The predicted structure for W3 was identical to that of CPI+.

The possibility that other changes between CPI+ and CPI− are responsible for the different biological properties of the two isolates. For example, no antigenic comparisons have been made between the F and L proteins of these viruses. Nevertheless, with regard to the differences in the P/V gene it is interesting to note that three of the four nucleotide substitutions between CPI+ and CPI− resulted in amino acid substitutions, whereas 14 nucleotide changes between W3 and CPI+ resulted in only five amino acid substitutions. Furthermore, according to both Chou & Fasman (1977) and Garnier et al. (1978) secondary structure protein prediction studies of the eight amino acid differences between W3 and CPI−, the only amino acid substitution that altered the predicted structure (by inducing helical turns) was the leucine to proline substitution which abolished the binding of the MAb P-k to native proteins (Fig. 5). All the other amino acid substitutions (including a leucine to proline substitution at amino acid position 50) had no effect on the predicted secondary structure of the protein and only minor effects on its hydrophilicity. Ironically however, using antigenic index predictions described by Jamerson & Wolf (1988), the amino acid substitution responsible for the loss of binding of MAb P-k changes the site from a predicted non-immunogenic to an immunogenic site. Whether the change in conformation predicted at the epitope binding site of MAb P-k in CPI− alters the function of the P or V protein in such a way that it influences the establishment of persistent infections remains only an intriguing possibility. To begin to address this question, further analysis of the functions of these proteins is required.

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References


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