Location of monoclonal antibody binding sites in the capsid protein of feline calicivirus

I. D. Milton,1 J. Turner,2† A. Teelan,1 R. Gaskell,2 P. C. Turner3 and M. J. Carter1

1 Division of Virology, School of Pathological Sciences, Medical School, Framlington Place, Newcastle upon Tyne NE4 2HH, 2 Department of Veterinary Pathology, University of Liverpool, Leahurst, Neston, Wirral, Merseyside L64 7TE, and 3 Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

We report the localization of three monoclonal antibody (MAb) binding sites in the capsid protein of feline calicivirus. Gene fragments were generated by restriction enzyme digestion or the polymerase chain reaction, and expressed as β-galactosidase fusion proteins in Escherichia coli. These chimeric molecules were screened using three MAbs. A non-neutralizing MAb recognized a region within 36 amino acids of the C terminus. Two neutralizing MAbs bound to a different region of 37 amino acids in the centre of the protein. Comparative sequence analysis shows this area to be the major variable region of the capsid protein.

Caliciviruses are positive-stranded RNA viruses of unique morphology. The virus surface appears to be covered in a series of cup-like depressions (reviewed by Carter & Madeley, 1987), for which the virus group is named (calix, a cup).

Feline calicivirus (FCV) is a ubiquitous pathogen of cats. The virus is associated with a variety of clinical symptoms, the most common being oral ulceration and upper respiratory tract infection (Hoover & Kahn, 1975; Povey & Hale, 1974), although complications such as the development of a polyarthritis (limping syndrome) have been reported (Studdert et al., 1970). FCV strains form a single serotype; these strains can be differentiated by using both polyclonal (Povey, 1974; Kalunda et al., 1975) and monoclonal antibodies (MAbs) (Tohya et al., 1991).

Recently, vaccination of domestic cats with a live-attenuated virus strain has been widely adopted. Such a vaccine promotes population immunity to a relatively restricted antigenic sub-population of the virus, and there is evidence that the emergence of restricted immunity among hosts has altered the relative preponderance of viruses in circulation to favour the emergence of variant strains (Knowles et al., 1990). Since FCV strains possess only a single capsid protein, the determinants of any such effects must reside in that molecule. In other RNA viruses capsid proteins can exert a strong influence on the tissue tropism and severity of disease resulting from virus infection (Spriggs et al., 1983; Davis et al., 1986). Consequently, antigenic variation driven by restricted immunity in the host population may have implications for virus pathogenesis. For these reasons it is important to understand the structural basis of antigenic variation in FCV.

The FCV capsid is constructed of a single major species of protein (cP62), synthesized as a precursor (cpP76) which is subsequently cleaved to produce the final form (Carter, 1989). We have recently reported the identification, cloning and sequencing of the capsid protein gene from a vaccine strain of FCV termed F9. The gene has also been expressed as a β-galactosidase fusion protein in Escherichia coli. The purified protein is immunogenic in mice, and reacts with feline antisera (Carter et al., 1992). We have also reported the production of two MAbs specific for the virus capsid (Carter et al., 1989). Of these, one (1G9) is capable of neutralizing the virus, whereas the other (1E1) is not. We have since derived several other MAbs including a second neutralizing antibody termed 4E7. These three antibodies are active in Western blotting procedures and react with the capsid protein, but not the non-structural proteins expressed in E. coli (Fig. 1).

We have exploited this reaction to examine antibody binding to regions of the capsid protein expressed from fragments of the gene. The minimum reactive segments should then localize the major determinants of antibody binding to the recombinant protein.

The capsid protein gene of feline calicivirus was
Western blot analysis of FCV-specific proteins expressed in E. coli. Bacteria carrying pEX plasmids containing virus cDNA sequences were induced to express the non-structural or structural protein genes from FCV as a β-galactosidase fusion protein, and analysed on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose and detected with appropriate antisera and conjugates as described (Carter, 1989). (a to d) Lanes 1, pEX containing the FCV non-structural protein gene; lanes 2, pEX containing the FCV capsid protein gene; lanes 3, pEX control lacking inserted virus-specific cDNA. (a) Protein detected with MAb 1El; (b) MAb IG9; (c) MAb 4E7; (d) serum from an immunized cat. F, β-Galactosidase fusion protein.

Prepared as a PstI-EcoRI fragment from clone pFC42. The derivation and sequencing of this clone have been described (Carter, 1990; Carter et al., 1992). This fragment does not contain the entire gene for the protein precursor, but does include all sequences expressed in the mature protein (Carter et al., 1992). The overhanging termini of the PstI-EcoRI fragment were repaired with T4 DNA polymerase and the fragment was then cleaved with HaeIII (Fig. 2). The resulting digest, containing 10 blunt-ended fragments (a to j), was ligated into the Smal site of the pEX1 to -3 family of expression vectors (Stanley & Luzio, 1984). This approach allows the insertion of DNA fragments in either orientation and in each of the three possible reading frames at the 3' end of the lacZ gene. Transcription of this gene is under the control of a λ phage promoter in these vectors, and is normally repressed by the action of a temperature-sensitive λ phage repressor protein provided by the host bacterium (strain popC 2136). Transcription of the gene occurs if transformed cells are grown at 42 °C, when the repressor is inactivated. Only one of the six possible orientations of each capsid gene fragment will be correct for translation, and will result in the expression of FCV capsid protein sequences fused to the C terminus of β-galactosidase. We obtained a library of approximately 700 clones which were induced to express β-galactosidase by temperature shift, and screened for reaction with each of the three MAbs by the method of Stanley (1983). Of the 700 clones, 1-1% reacted with MAbs 1G9 and 4E7, and an additional 1% of clones were recognized only by MAb 1E1. The size of the library size exceeded the theoretical number of clones required to ensure expression of all 10 fragments (Maniatis et al., 1982), and the observation of multiple expressing clones supports this.

Plasmid DNA was extracted from all clones and identified by sequencing the vector directly using the synthetic oligonucleotide 5' CCATCGCCATCTGCTGC 3', which binds at position 2912 within the lacZ gene and primes DNA synthesis across the vector multiple cloning site and into inserted DNA (Carter et al., 1992). In this way all clones reacting with MAbs 1G9 and 4E7 were found to contain fragment g, and all those recognized by MAb 1El contained fragment j (Fig. 2). Clones were also checked for size of inserted DNA and absence of an internal HaeIII site in order to confirm complete digestion. Two clones recognized by MAb 1E1 contained inserts larger than fragment j, and these also possessed an internal HaeIII site. The second fragment was inserted distal to fragment j and was different in both cases. These were concluded to be recombinants which had incorporated two restriction fragments during ligation. Consequently the contribution of these distal sequences to antibody recognition was discounted.

The sequences recognized by each antibody were further investigated by dissection of the expressed DNA fragments. The fragment binding MAb 1E1 contained a XhoI site near the 5' end; this was used to subdivide the fragment and localize the binding site to one piece (Fig.
Table 1. Identification of expressed gene fragments and corresponding amino acids

<table>
<thead>
<tr>
<th>Restriction fragment</th>
<th>Nucleotides*</th>
<th>Amino acids</th>
<th>Reactivity with MAb</th>
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<tbody>
<tr>
<td>g (HaeIII–HaeIII)</td>
<td>1261–1591</td>
<td>421–530</td>
<td>1G9, 4E7</td>
</tr>
<tr>
<td>j (HaeIII–EcoRI)</td>
<td>1625–2015</td>
<td>543–671</td>
<td>1E1</td>
</tr>
<tr>
<td>HaeIII–XhoI</td>
<td>1625–1682</td>
<td>543–562</td>
<td>None</td>
</tr>
<tr>
<td>XhoI–EcoRI</td>
<td>1683–2015</td>
<td>562–671</td>
<td>1E1</td>
</tr>
</tbody>
</table>

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<tr>
<th>PCR</th>
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<th>Amino acids</th>
<th>Reactivity with MAb</th>
</tr>
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<tbody>
<tr>
<td>Primers 1 + 2</td>
<td>1259–1468</td>
<td>422–489</td>
<td>1G9, 4E7</td>
</tr>
<tr>
<td>Primers 1 + 4</td>
<td>1259–1374</td>
<td>421–458</td>
<td>1G9, 4E7</td>
</tr>
<tr>
<td>Primers 1 + 3</td>
<td>1259–1902</td>
<td>421–634</td>
<td>1G9, 4E7</td>
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<table>
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<th>Primers used</th>
<th>Sequence</th>
<th>Binding site*</th>
<th>Sense</th>
</tr>
</thead>
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<tr>
<td>Primer 1</td>
<td>5’ GGCTGACACCACAAT 3’</td>
<td>1259–1274</td>
<td>+</td>
</tr>
<tr>
<td>Primer 2</td>
<td>5’ CAGTGATGATAAGG 3’</td>
<td>1454–1468</td>
<td>–</td>
</tr>
<tr>
<td>Primer 3</td>
<td>5’ ACAAAAGAGACCACCATC 3’</td>
<td>1883–1902</td>
<td>–</td>
</tr>
<tr>
<td>Primer 4</td>
<td>5’ CGTATATATCACCAGTGTC 3’</td>
<td>1354–1374</td>
<td>–</td>
</tr>
</tbody>
</table>

* All nucleotide positions are given relative to the initiation codon for the capsid gene, the A residue of which is position 1.

2). Fragment g contained no easily usable restriction enzyme sites, and further dissection of both fragments was subsequently performed using the polymerase chain reaction (PCR) to amplify selected regions. Primers were chosen so as not to interrupt hydrophilic peaks predicted in the protein by the method of Kyte & Doolittle (1982) and incorporated into the SOAP and ANTIGEN programs of PC Gene (Intelligenetics). Such hydrophilic sections of protein are known to be associated with immunogenicity (Hopp & Woods, 1981).

Each amplified fragment was initially cloned into the SmaI site of the vector pTZ18R (Pharmacia) and sequenced before being subcloned into the expression vector. Most fragments were relatively short (< 500 bp) and could be sequenced completely from the single primer site in this vector. Those which could not be sequenced by this method were reversed by subcloning into pTZ19R and sequenced in the opposite orientation. This procedure confirmed the identity of each fragment, verified the sequences of both termini and identified the reading frame required for expression in the pEX vector. Cloned PCR fragments were excised and recloned into the appropriate pEX expression vector and recombinants were screened for reactivity with each of the three MAbs. Table 1 lists the primers used and amplification products formed in PCR. The results of this analysis are summarized in Fig. 2 and Table 1, which illustrate the sections of the gene expressed and the amino acids involved, and the reactivity of the fusion proteins formed. The binding site of MAb 1E1 was localized to 36 amino acids between positions 636 and 671, and that of MAbs 1G9 and 4E7 to 37 amino acids between positions 422 and 458.

Recently the capsid protein sequences of two other FCV strains have been determined (Tohya et al., 1991; Neill et al., 1991). The sequences of all three FCV capsid proteins were compared using the CLUSTAL program in PC Gene. Conservation between these strains is high (84-2%), but some areas appeared to be less conserved than others. This variation is clearly demonstrated in a variation plot; the method used in this analysis is described in the legend to Fig. 3. This reveals two regions of variation, one located at the N terminus and one between residues 400 and 500, although this latter region is divided into two by a small, highly conserved section. The site at which the precursor protein is cleaved (residue 124) is indicated (Carter et al., 1992). Thus the variation at the N terminus is not represented in the mature virus. The location of the MAb binding sites identified is illustrated in Fig. 3. Both neutralizing MAbs recognize the variable section of the protein, whereas the non-neutralizing antibody recognizes a separate region at the C terminus.

The MAbs used were all raised against the vaccine strain F9. To differentiate between the binding sites of MAbs 1G9 and 4E7 we investigated cross-reactivity with
Fig. 3. Variation plot of aligned FCV capsid sequences. The aligned proteins were divided into windows of 10 amino acids, each overlapping by five residues. The number of positions at which at least one of the strains differed was determined in each window and plotted against the amino acid sequence. A score of 0 indicates absolute conservation between strains; a maximum variation score of 10 is possible in this analysis.

Fig. 4. Cross-reactivity of the MAbs. CRFK cells were infected with FCV strain F9 (to which the antibodies were raised) or CFI/68, sequenced by Neill et al. (1991). Infected and mock-infected cells were then lysed and transferred to nitrocellulose by dot blot. The filter was then divided and stained by different methods. In each strip: 1, mock-infected cell lysate; 2, strain CFI/68-infected cell lysate; 3, strain F9-infected cell lysate. Strips were stained as follows: (a) total protein stain (India ink); (b) serum from a vaccinated cat; (c) MAb IE1; (d) MAb 4E7; (e) MAb 1G9.

strain CFI/68 (used by Neill et al., 1991) which was kindly provided by Dr W. Mengeling (National Animal Disease Centre, USDA-ARS, Ames, Ia., USA). We compared the reactivity of the three MAbs by protein dot blot (Fig. 4). Whereas MAbs 4E7 and 1E1 reacted with both strains, MAb 1G9 recognized only strain F9. Sequence comparison of these two strains in the capsid protein region of binding identified in Fig. 2 showed that the region within which MAb 1E1 binds is conserved: only two amino acids differ between strains CFI/68 and F9 (Fig. 5). This high level of identity explains why MAb 1E1 cross-reacts with the two strains. The region within which MAbs 1G9 and 4E7 bind is 37 amino acids long (Fig. 5). Of the first 27 amino acids, only three differ between strains F9 and CFI/68, and of the last 10 amino acids six are different. We suggest that MAb 1G9 is more likely to bind in the variable region and thus differentiate between FCV strains. MAb 4E7 could bind in the conserved region.

Higher level protein structure may bring regions distant in primary sequence together through intra- and intermolecular associations. Indeed, the binding sites of MAbs 1G9 and 1E1 are known to be close in the assembled protein because binding of one antibody excludes the other (Carter et al., 1989). Consequently, these experiments do not necessarily provide complete information on the epitopes of the virus. Contributions to the structure of an epitope from such non-contiguous sequences may be important in stimulating the immune response. This approach can only identify regions with a sufficiently high affinity for the antibody to bind stably to the prokaryotic product under the conditions of blotting, and these regions may form only part of an epitope. However, if other regions of the protein are
involved in the structure of an antigenic epitope, their affinity for the MAbs elicited is too low to permit detection by this technique.

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References


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