A Neutralizing Epitope on Human Rhinovirus Type 2 Includes Amino Acid Residues between 153 and 164 of Virus Capsid Protein VP2

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SUMMARY

Use has been made of a monoclonal antibody (designated 8F5) to map a neutralizing epitope on the viral capsid protein VP2 of human rhinovirus 2 (HRV2). This antibody which was raised against the native virus, neutralizes HRV2 and is also capable of recognizing denatured VP2 on Western blots. To examine the binding site of 8F5, VP2 of HRV2 was expressed in Escherichia coli. Deletions starting at the 3' end were then introduced into the gene for VP2 using Bal-31 nuclease. Polypeptides shortened at the carboxy terminus of VP2 were obtained from the deletions and were blotted onto nitrocellulose. The samples were then probed with monoclonal antibody 8F5. Recognition by 8F5 was maintained as long as the expressed polypeptide contained the VP2 sequence up to amino acid 164 or beyond. However, when the VP2 sequence was truncated to amino acid 153 or less 8F5 was no longer able to bind. The neutralization epitope (or part of it) recognized by 8F5 on VP2 is therefore located between amino acids 153 and 164.

INTRODUCTION

The majority of upper respiratory tract infections collectively known as the common cold are caused by human rhinoviruses (Gwaltney, 1975). The existence of more than 100 antigenically distinct serotypes is a major factor in precluding the acquisition of immunity to rhinovirus infection (Fox, 1976; Stott & Killington, 1972). Rhinoviruses form the largest genus of the family Picornaviridae and possess the characteristic physical and structural properties of viruses from this family. In short, picornaviruses comprise an icosahedral capsid, made up of 60 copies each of four virus-encoded proteins (VP1 to VP4), which surrounds a single-stranded, positive sense RNA molecule of between 7100 and 7500 nucleotides in length (Cooper et al., 1978; Melnick, 1980).

In spite of the economic and medical importance of the high level of serotypic diversity found in human rhinoviruses, its molecular basis is not understood. Nevertheless, it has been demonstrated that different rhinovirus strains use one of only two cellular receptors (Abraham & Colonno, 1984). According to their specific receptor binding, they can be divided into a major and a minor group. Recently, however, work from several laboratories has considerably enlarged our knowledge of the molecular biology of these viruses; the complete nucleotide sequences of human rhinovirus 14 (HRV14) and human rhinovirus 2 (HRV2) have been determined and the crystal structure at 0.3 nm resolution of HRV14 has also been elucidated (Callahan et al., 1985; Rossmann et al., 1985; Skern et al., 1985; Stanway et al., 1984). Furthermore, Sherry & Rueckert (1985) and Sherry et al. (1986) have mapped a series of neutralization-resistant mutants with monoclonal antibodies raised against HRV14 and have shown that HRV14 possesses at least four epitopes involved in neutralization. These epitopes all lie at sites which protrude from the virus surface as seen in the three-dimensional structure of the virus particle.

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In view of the antigenic diversity among the human rhinoviruses it is also of interest to gain insight into the structure of HRV2, particularly as HRV14 binds to the major group receptor whereas HRV2 binds to the minor group receptor. For this purpose monoclonal antibodies were raised against native HRV2. One of these, designated 8F5, was found to neutralize HRV2 and to recognize a viral capsid protein (VP2) on Western blots. In this report we describe the localization of the binding site of monoclonal antibody 8F5 to a region of 12 amino acids on VP2. The same approach was taken previously by Wychowski et al. (1983) to identify an antigenic site on VP1 of poliovirus. The properties of some of the other monoclonal antibodies directed against HRV2 will be described elsewhere by C. Neubauer et al.

**METHODS**

**Materials.** Restriction enzymes were from New England Biolabs or Boehringer Mannheim; bacterial alkaline phosphatase (BAP) was from Bethesda Research Laboratories; nuclease Bal-31 was from New England Biolabs; T4 polynucleotide kinase was from P-L/Pharmacia. Nitrocellulose (0.45 μm pore) was purchased from Schleicher & Schuell; [γ-32P]ATP was from Amersham International; peroxidase-conjugated rabbit anti-mouse IgG was obtained from Bio-Yeda; o-dianisidine was from Sigma. All other chemicals were of reagent grade.

**Bacterial strains and plasmids.** pEx34b is a 3.0 kb plasmid containing the ribosome-binding site and the first 150 amino acids of the MS2 polymerase gene which have been placed under the control of the λ P1 promoter. A small polylinker with sites for EcoRI, BamHI and HindIII enables suitable DNA fragments to be introduced in phase after the MS2 polymerase (pol) sequence. The plasmid also contains the origin of replication and the gene for ampicillin resistance (Amp') from pBR322. Escherichia coli W6(λ) expresses constitutively the gene for the wild-type repressor and E. coli 537 bears the ci853 λ repressor mutation (inactive at 42 °C) on a plasmid conferring kanamycin resistance. This system has been described elsewhere (Strebel et al., 1986). Bacterial strains and expression plasmids were kindly provided by Drs E. Pfaff, E. Beck and H. Schaller (Heidelberg, F.R.G.).

**DNA manipulations and production of Bal-31 deletions.** Plasmid DNA was prepared by the method of Birnboim & Doly (1979). Cleavage of DNA by restriction enzymes was carried out under conditions described by the suppliers. Digestion of plasmid pEx34b-969 DNA by Bal-31 nuclease was performed as follows. 770 ng of pEx34b-969 which had been cut at the single BssHII site was incubated with 0.75 units of Bal-31 nuclease in 20 mM-Tris-Cl pH 8.0, 600 mM-NaCl, 12 mM-MgCl2, 12 mM-CaCl2 and 1.0 mM-EDTA. Samples were removed at various time intervals (see Results) and the reaction was stopped by the addition of EDTA to 30 mM. The DNA was recovered by ethanol precipitation and the plasmids were recircularized with T4 DNA ligase in a reaction containing 170 ng digested pEx34b-969 DNA, 200 units T4 DNA ligase, 10 mM-Tris-Cl pH 7.5, 6 mM-MgCl2, 6 mM-2-mercaptoethanol and 1 mM-ATP. Incubation was overnight at 15 °C. The mixture was used directly to transform competent E. coli W6(λ). DNA sequencing was performed as described by Maxam & Gilbert (1980).
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Expression of fusion proteins in E. coli. For expression, plasmid DNAs of interest were isolated from transformed E. coli W6(λ) and introduced into E. coli 537 cells. Induction of the λ P1 promoter was as follows. Cells were grown at 28 °C under selection (100 μg/ml ampicillin, 25 μg/ml kanamycin) to high density. The cultures were then diluted to 5 vol. with prewarmed L broth (without antibiotics) and were incubated at 42 °C with vigorous shaking. After 2 h, cells from 1 ml of culture were harvested by centrifugation and resuspended in 500 μl cold sonication buffer (50 mM-Tris-HCl pH8.0, 150 mM-NaCl and 1 mM-EDTA). Disruption of the cells was achieved with an MSE ultrasonic power using three bursts of 5 s each. Forty-five s intervals were allowed between bursts to prevent overheating. Insoluble material was collected by centrifugation for 2 min in an Eppendorf centrifuge and the pellets were dissolved in 200 μl sample buffer (Laemmli, 1970). After heating at 95 °C for 4 minutes, 10 μl of the samples was examined on a 10% polyacrylamide gel containing SDS as described (Laemmli, 1970). Control experiments showed that all expressed proteins were insoluble in sonication buffer. Bands were either stained with Coomassie Brilliant Blue or were blotted onto nitrocellulose using the method of Burnette (1981). Blotting was carried out at 70 V for 4 h in a Bio-Rad protein blot chamber in the presence of 0.1% Empigen (Mandrell & Zollinger, 1984).

Examination of the antigenic properties of expressed proteins after blotting onto nitrocellulose with monoclonal antibody 8F5 was performed in the following manner. The filters were incubated overnight at room temperature with 10 μl of blocking solution [phosphate-buffered saline (PBS) containing 1% bovine serum albumin, 1% Tween 20 and 10% calf serum], and were then incubated with 2 μl of blocking solution containing 10 μl ascites fluid from monoclonal antibody 8F5 for 2 h at room temperature. After rinsing well with water, the filters were washed three times for 15 min each with PBS containing 1% Tween 20. Peroxidase-conjugated rabbit anti-mouse IgG was diluted 1:1000 in 5 ml of blocking solution and the filters were incubated in this solution for 3 h. The above washing process was repeated and the bound peroxidase-conjugated antibody detected using o-dianisidine as colour reagent; the filters were bathed in 50 ml PBS containing 0.025% hydrogen peroxide and 0.5 mg/ml o-dianisidine for 10 min at room temperature; the reaction was stopped by extensive rinsing with water. Proper controls were carried out to ensure that a negative signal was not due simply to poor transfer.

Monoclonal antibodies. BALB/c mice were injected intraperitoneally with 5 to 10 μg purified HRV2 emulsified in complete Freund's adjuvant and boosted on days 27, 49, 100, 117 and 118 with the same amount of virus in incomplete adjuvant. One day after the last inoculation, spleens were removed and cells fused with NS-1 myeloma cells following published protocols (Galfré & Milstein, 1981). The supernatants of cell culture wells containing growing colonies of fused cells were examined for the presence of virus-neutralizing antibodies by microneutralization tests as follows. Fifty μl hybridoma supernatants in 96-well microtitre plates were mixed with 1000 p.f.u. HRV2 in 50 μl minimal essential medium containing 30 mM-MgCl2 and 2% foetal calf serum and incubated for 1 h at 34 °C in 5% CO2. To each well was added 3 x 104 HeLa Ohio cells (Flow Laboratories) and the plates were incubated as above. After 48 h the plates were stained with 0.1% crystal violet in 50% ethanol. Wells containing intact cell layers were scored as positive. Hybridomas were cloned three times by limiting dilution and used for production of ascites. Under these conditions a 1:1000 dilution of the ascites fluid resulted in 50% neutralization.

RESULTS

Expression of VP2 as a fusion protein in E. coli

The strategy employed to express the capsid protein VP2 in E. coli is shown in Fig. 1. Use was made of pHRV2-969; this plasmid contains a 1.1 kb HRV2 cDNA insert with the genes for the carboxy-terminal half of VP4, all of VP2 and the first 60 amino acids of VP3 cloned into the PstI site of pUC9. To isolate a fragment containing the gene for VP2 which would be suitable for insertion into the expression vector pEx34b, plasmid HRV2-969 was cut at the EcoRI site within the gene for VP4 (nucleotide 744 on the HRV2 map; see Skern et al., 1985) and at the HindIII site in the polylinker at the 3' end of the insert. This 1.1 kb EcoRI-HindIII fragment was then cloned into pEx34b which had been cut with EcoRI and HindIII. Analysis of the sequence had shown that such a construction should enable translation to continue from the MS2 polymerase gene into the HRV2 genes in the correct phase. Expression of induced proteins from pEx34b-969 was then examined in E. coli 537 cells as described in Methods. Fig. 2(a) shows a Coomassie Brilliant Blue-stained polyacrylamide gel of the proteins found in the pellets obtained after sonication of cells which had been induced for 2 h at 42 °C. It can clearly be seen that there was a strong protein band of apparent mol. wt. 58000 present in the cells containing pEx34b-969. Control experiments (data not shown) revealed that this band appeared only on induction at 42 °C. Furthermore, none of this protein was found in the supernatant following sonication. The mol. wt. of 58000 is in good agreement with the number of amino acids expected to be present in the expressed protein.
Fig. 2. Expression of capsid protein VP2 as a fusion protein in *E. coli*. (a) Coomassie Brilliant Blue-stained SDS-polyacrylamide gel of the material insoluble in sonication buffer from cells containing the plasmids pEx34b-969 (lane 2) or pEx34b (lane 3), after induction at 42 °C. The purified virus (lane 1) was included as a positive control for the subsequent immunoblot. (b) Immunoblot of the polyacrylamide gel from (a). The gel was blotted onto nitrocellulose and probed with monoclonal antibody 8F5. Of the viral proteins only VP2 was recognized. The protein appearing on induction of pEx34b-969 at 42 °C gave a strong signal, whereas no signal was observed from the proteins obtained using the vector alone.

Recognition of the induced protein from pEx34b-969 by monoclonal antibody 8F5

In order to demonstrate that the 58 000 mol. wt. protein expressed from plasmid pEx34b-969 under the control of the λ P_l promoter contained VP2-specific amino acid sequences, proteins in a polyacrylamide gel identical to that in Fig. 2(a) were blotted electrophoretically onto nitrocellulose and incubated with the neutralizing monoclonal antibody 8F5. The result of this blotting is shown in Fig. 2(b). The first lane contains capsid proteins of purified HRV2; it can be seen that only VP2 was recognized by 8F5. The 58 000 mol. wt. protein present in extracts from
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Fig. 3. (a) Coomassie Brilliant Blue-stained SDS-polyacrylamide gel of proteins expressed upon induction at 42 °C, from Bal-31 deletion plasmids derived from pEx34b-969. Lanes labelled 2, 4, 6, 8 and 10 refer to the incubation times in minutes with Bal-31 nuclease; the other lanes show proteins from other clones obtained from a similar deletion experiment (402, 513 and 601 from the 4 min, 5 min and 6 min time points respectively). (b) Immunoblot of the polyacrylamide gel from (a).

cells harbouring pEx34b-969 gave a strong signal, thus confirming that it contained HRV2 sequences (lane 2). No signal was observed from proteins obtained from cells containing only the parental plasmid pEx34b (lane 3).

Determination of the binding site of monoclonal antibody 8F5

The technique previously described by Wychowski et al. (1983) was employed to determine the region of VP2 recognized by monoclonal antibody 8F5. The plasmid pEx34b-969 was opened at the single BssHII site and digested in both directions with Bal-31 exonuclease; aliquots were removed after 2, 4, 6, 8 and 10 min. The shortened plasmids were recircularized and used to transform competent E. coli 537 cells. The proteins expressed were examined on 10% SDS-polyacrylamide gels. Fig. 3(a) shows the result of a typical experiment; the protein pattern from one representative of each sample is shown. Induction of expressed proteins could clearly be seen at the 2, 4, 6 and 8 min time points. No induced protein was visible in the 10 min sample, probably because the protein was too small to be seen in this gel system.

To examine the ability of monoclonal antibody 8F5 to bind to the proteins expressed from the deleted plasmids, a gel identical to that in Fig. 3(a) was blotted onto nitrocellulose and probed with the antibody. The result is shown in Fig. 3(b). A strong signal was seen from the proteins from the 2 and 4 min time points; the smaller bands seen in the 2 min sample probably resulted from proteolytic degradation. However, no signal was observed for the proteins from the 6, 8 and 10 min time points; nevertheless, these proteins could be transferred efficiently as indicated in control experiments (data not shown). This is taken to indicate that these proteins no longer
contained the binding site for the monoclonal antibody 8F5; in this experiment loss of the binding site therefore occurred between 4 and 6 min after the start of the Bal-31 digestion.

To ensure that enough deletions were available to localize precisely the binding site, a second Bal-31 nuclease digestion was performed. Conditions were identical to those used in the first experiment except that samples were taken only after 4, 5 and 6 min incubation. The proteins expressed from a further 15 deleted plasmids were examined in a similar manner for the presence of the 8F5 binding site. The proteins from three such selected plasmids and the corresponding blots are also shown in Fig. 3 (a) and (b) respectively. Two of these plasmids, 402 and 601, expressed proteins which still gave a signal with monoclonal antibody 8F5, whereas that derived from plasmid 513 was no longer recognized. In the case of 402, two defined bands were detected on challenge with monoclonal antibody 8F5. As no evidence of smearing towards low molecular weight products was evident, the presence of the two bands was more likely due to leakiness of the stop codon than to proteolytic degradation. Furthermore, it can also be seen that there were differences in size between proteins in samples taken at the same time point. The main reason for this is that Bal-31 nuclease digests the DNA in both directions; after the digestion has been stopped, the two ends are religated. Thus, transcription continues past the end of the truncated VP2 gene into VP3 and/or sequences from pBR322. Thus, the size of the protein obtained will depend on how much of the RNA is translated before a stop codon is encountered. This will be in turn determined by the extent of the digestion into this region and the reading frame after the religation.

The behaviour of the proteins expressed from eight of the plasmids is listed in Fig. 4, together with the position of the carboxy end of the truncated VP2 generated by the deletions. These were mapped by restriction analysis. For instance, all plasmids containing the SspI site at position 1364 of the HRV2 map (corresponding to amino acid 183 of VP2) gave rise to proteins which were recognized by the antibody 8F5, whereas plasmids lacking the RsaI site at position 1177 (amino acid 120 of VP2) produced a protein not recognized by the antibody. Thus, it was inferred that the 8F5 binding site was encoded between these two restriction sites. Nucleotide sequencing was then employed to define exactly the limit of each deletion; this revealed that only 12 amino acids separated the carboxy end of the truncated VP2 of the protein induced from plasmid 532 from that of plasmid 601. As the protein from plasmid 532 was not recognized by
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HRV2 VP2

1 100 200 261

RsaI SspI BssHII

8F5 binding site

1240 1280 1320

aac gtt ggt tac aac cac cca ggt gaa aca ggc agg gaa gtt gac gaa aga aga tlg aat cct gct ctc cca cct

N ¥ G Y N Y T H P G E T G R E V K A E T R L N P D L O P

622 513 527 532 601 402

Fig. 5. Diagrammatic representation of the position of the 8F5 binding site on the viral capsid protein VP2. The nucleotide and amino acid sequences around the binding site are shown with the exact position of each deletion marked underneath with an arrow. The numbers (1 to 261) above the line refer to the amino acid sequence of VP2, those under the expanded binding site to the nucleotide sequence of HRV2 taken from Skern et al. (1985).

monoclonal antibody 8F5 whereas that of 601 was, these 12 amino acids (or a subset thereof) represent an essential part of the binding site on capsid protein VP2 for monoclonal antibody 8F5. Fig. 5 shows the location of this site on the capsid protein, as well as its amino acid sequence and the nucleotide positions of the individual deletions.

DISCUSSION

The elucidation of the molecular basis of serotypic diversity is an important goal in rhinovirus research. How do the 115 serotypes differ from one another, whilst at the same time retaining the biological and physical characteristics of a rhinovirus? Recent work with HRV14 reported by Rossmann et al. (1985) and Sherry et al. (1986) has demonstrated the presence of four neutralization immunogens. These immunogens, designated NIm-IA, NIm-IB, NIm-II and NIm-III, protrude from the surface of the virus and are distinct entities. However, as mentioned above HRV14 and HRV2 belong to different groups in terms of their receptor specificity (Abraham & Colonno, 1984). Furthermore their capsid proteins are only about 50% homologous at the amino acid level. It is therefore not clear a priori whether immunogens lying on the surface of HRV2 will exhibit a similar pattern in HRV14.

To begin to answer this question, an analysis of the binding site of the neutralizing monoclonal antibody 8F5 on the viral capsid protein VP2 was undertaken. As this antibody is capable of recognizing the denatured protein, it was likely that only primary structure determinants were involved and that consequently a neutralizing immunogen on VP2 could be defined. Analysis of the binding properties of proteins expressed from plasmids containing a truncated gene of VP2 showed that the binding site of 8F5 was maintained provided the deletion from the carboxy end did not extend further than amino acid 164. However, no binding was observed as soon as the deletion reached amino acid 153. It can therefore be concluded that the C-terminal boundary of the amino acids constituting this immunogen must lie within these 12 amino acids. The lack of a suitable restriction site in the sequence between the N terminus of VP2 and this region precluded a definition of the N-terminal boundary of the 8F5 binding site.

In HRV14, the mutations that defined neutralization immunogen II (NIm-II) occurred between amino acids 158 and 162 on VP2 (Sherry et al., 1986). It is therefore clear that the binding site of 8F5, lying between amino acids 153 and 164 on HRV2 is analogous to NIm-II of HRV14. The fact that the 8F5 antibody recognizes its binding site even after denaturation of the
protein by SDS makes it highly unlikely that binding is conformationally dependent on amino acids 153 to 164. Comparison of the amino acid sequences 153 to 164 from HRV2 and HRV14 reveals that only one amino acid, glycine 153, is conserved between the two serotypes, an observation which is not unexpected for a region involved in defining the antigenic determinants.

However, both regions contain eight hydrophilic residues, five of which are charged in HRV2 and two in HRV14. Rhinoviruses have been divided into subgroups on the basis of the low degree of antigenic cross-reactivity with each other (Cooney et al., 1982; Gwaltney, 1975), and HRV2 and HRV14 have been placed in different subgroups. It will therefore be of interest to compare the NIm-IIs of human rhinovirus serotypes which belong to the same subgroup to see whether more amino acids are conserved in this region. Such conservation may be a factor in determining the extent of cross-reactivity.

In summary, the work in this paper shows the localization of an essential part of the binding site of a neutralizing monoclonal antibody to a region of 12 amino acids of the viral capsid protein VP2 of HRV2. This region is analogous to a previously mapped neutralizing immunogen of HRV14. The characterization of other monoclonal antibodies raised against HRV2 will present a more detailed picture of the antigenic properties of this virus; experiments to this end are currently in progress.

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