Linear antigenic and immunogenic regions of the respiratory syncytial virus P protein

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Three linear antigenic regions on the P protein from human respiratory syncytial virus (RSV) subgroup A (strain A2) were represented by peptides that reacted with monoclonal antibodies and with sera from humans with recent or previous RSV infection. The determinants were localized within three hydrophilic regions of the P protein: Pro91 to Asp110, Ser161 to Lys180 and Glu221 to Phe241. The role of individual amino acids in the epitopes defined by monoclonal antibodies was determined. Two monoclonal antibodies reacting with the same antigenic site were found to detect epitopes that had different amino acid dependencies. Rabbit hyperimmune sera raised against selected peptides specifically precipitated different forms of the P protein from RSV-infected 35S-labelled cell extracts in a radioimmune precipitation assay. These findings have implications for forthcoming structural-functional studies of RSV capsid component interactions and also for serological diagnosis of RSV infection.

Introduction

Respiratory syncytial virus (RSV) is a negative enveloped ssRNA virus belonging to the genus Pneumovirus in the family Paramyxoviridae. RSV is the single most important viral agent that causes severe lower respiratory tract disease (bronchiolitis and pneumonia) in infants and children (McIntosh & Chanock, 1985). Molecular studies of this virus, for example by the use of synthetic peptides representing T and B cell epitopes of the virus proteins, offer both an insight into RSV immunobiology and also provide a basis for vaccine development.

The phospho (P) protein of RSV together with nucleocapsid (N), large (L) proteins and viral genomic RNA build up the nucleocapsid core of the virion (Collins, 1991) which is the transcription complex of the virus, as shown in recent studies (Barik, 1992; Huang et al., 1993). By analogy to other paramyxoviruses (Ryan & Kingsbury, 1988; Collins, 1991), as well as to the well-studied prototype rhabdovirus vesicular stomatitis virus (VSV) (reviewed by Banerjee & Barik, 1992), it is believed that the P protein plays a key role in the regulation of the transcription-replication process. The aim of our study was to identify antigenic regions on the RSV P protein and to map epitopes within these sites at the level of individual amino acids. Immunodominant epitopes are involved in protein–protein interactions within protein complexes, for instance the glycoprotein complex of human immunodeficiency virus (HIV) (Neurath et al., 1992). Peptides that mimic regulatory protein sequences, e.g. the VSV P protein, can interfere with the viral transcription process (Yamashita & Kawai, 1990). Hence peptides, anti-peptide animal sera and monoclonal antibodies (MAbs) may be suitable tools for structural–functional studies of the replicative complex of RSV. Definition of the critical antigenic and immunogenic sites furthermore increases the understanding of the structural–functional characteristics of individual proteins and hence facilitates studies of specific reactions between different viral proteins. In addition, the same reagents may be used as diagnostic tools for site-directed subgroup-specific serology of RSV (Norrby et al., 1987; Åkerlind-Stopner et al., 1990).

In the present study, we identified three antigenic regions by use of synthetic overlapping peptides that represented the RSV P protein. The peptides reacted with MAbs and with a panel of 20 RSV-positive sera samples derived from convalescing patients. All three sites are localized within three conserved regions of the P protein (Mallapeddi & Samal, 1992), i.e. Pro91–Asp110, Ser161–Lys180 and Glu221–Phe241. Within these regions the epitope dependence on individual amino acids was determined. Rabbit polyclonal sera raised against antigenic peptides specifically precipitated different forms of the P protein (Caravokyri et al., 1992) from RSV-infected 35S-labelled cell extracts in a radioimmune precipitation assay (RIPA).
peptides demonstrated 55 to 97% homogeneity, as revealed by HPLC on a Nova-pak C_8 column (Waters Associates). The amino acid composition was confirmed by amino acid analysis. A cysteine residue was coupled to the C terminus of those peptides which lacked this amino acid in their sequence, in order to enable coupling to keyhole limpet haemocyanin (KLH) as described (Sambrook et al., 1989).

**MAbs.** Antibodies specific for the P protein and N protein, used as a control, were used. One group of MAbs was produced using the Long strain of RSV, a prototypic strain of subgroup A (Mufson et al., 1985). The following antibodies from this group were used: anti-P protein (anti-P) antibody for epitope P1-C771; anti-NP antibodies for epitopes NP1-C797 and -B90. Other anti-P-specific MAbs raised against subgroup A strains that were used were M189, M1482, M1485, M1610, M1624, M1627, M1633 and M1672, kindly provided by Dr C. Orvell, Stockholms Mikrobiologiska Central Laboratorium, Stockholm, Sweden. Additional subgroup A RSV P-specific MAbs RS201, RS202A, RS203 and RS205 were generated in the Department of Virology, University of Turku, Finland (Warts, 1992). A second group of MAbs was produced in the Swedish Institute for Infectious Disease Control (Stockholm, Sweden) using RSV WV4843, a subgroup B strain, as published (Orvell et al., 1987). The following antibodies from this group were used: anti-P antibodies specific for three epitopes, i.e. P1-8268, P2-9178 and P3-9516. Additional anti-P MAbs that were generated against a subgroup B strain were 3-5 and 4-14 (Gimenez et al., 1984), kindly provided by Dr H. B. Gimenez, Department of Biochemistry, University of Aberdeen, U.K. Finally, other MAbs used as controls were anti-NP specific for parainfluenzae virus type 3, C106 (kindly provided by C. Orvell) and anti-human papillomavirus type 16 (HPV-16) peptide 245:11E3 (a kind gift from Dr J. Dillner, Department of Virology, Karolinska Institute, Stockholm, Sweden).

**Animal sera.** Rabbits were injected subcutaneously with 100 μg of KLH-conjugated synthetic peptides in Freund’s complete adjuvant, and then with two injections of the same peptides in Freund’s incomplete adjuvant at 3-week intervals. The animals were bled 10 days after the final injection. Pre-immune and hyperimmune rabbit sera were collected and stored at −20 °C until use.

**Human sera.** Twenty RSV-positive sera samples derived from convalescing patients (HCS), with clearly demonstrable titres of RSV IgG in enzyme immunoassays (EIA) based on RSV nucleocapsid antigen (Waris, 1992) and seven paired human serum samples from patients with acute RSV infection (primarily diagnosed by detection of antigen in nasopharyngeal aspirates) were collected. One negative human serum, no. 92/2159, STII, that was derived from an infant of 8 months and had a mean A_492 value of < 0.2 at 1:100 dilution in a peptide ELISA was used as a negative control.

**ELISA.** ELISA with tissue culture-derived RSV antigen and peptides was performed as previously described (Åkerlind-Stopner et al., 1990) with the following minor changes: the plates were 96-well polystyrene plates [MaxiSorpF96 Nunc-immuno plates (Nunc) or half-area (A/2) plates (Costar)]; 1% fat-free milk was used as the blocking solution, incubated for 90 min at 37 °C; anti-human, anti-rabbit and anti-mouse peroxidase conjugates (Southern Biotechnology) were diluted 1:1000 in ELISA buffer; the substrate was 0.4 mg/ml o-phenylenediamine hydrochloride with 0.003% H_2O_2 in substrate buffer, and the enzymatic reaction was stopped after 30 min by the addition of an equal volume (50 μl) of 2.5 M H_2SO_4 and the A_492 was measured. The results were expressed either as A_492 (mean of three determinations) or, for substituted peptide analogues, as the percentage of binding. The titres of immune anti-peptide sera were calculated as the highest dilution that gave a mean A_492 value that exceeded the “cut-off” value by more than 0.2 absorbance units, i.e. exceeded the mean of three determinations of A_492 performed in wells with the same dilution of pre-immune sera plus two standard deviations. The peptide competition ELISA was

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**Fig. 1. ELISA with 20 amino acid long peptides representing the P protein and murine anti-RSV P protein MAbs (at a dilution of 1:1000): C771 (a), RS203 (b), RS205 (c) and M1624 (d). A_492 is shown after subtraction of the A_492 of the control wells (see Methods).**

**Methods**

**Virus.** RSV prototype strain A2 (subgroup A) was passaged in Vero cells and stored at −70 °C before use.

**Cells.** Vero cells were grown in Eagle’s MEM supplemented with 3% fetal calf serum (FCS) that had been inactivated by heating at 56 °C for 30 min. Virus was passaged as described elsewhere (Åkerlind et al., 1988) in nearly confluent monolayer cultures at 10 pfu/cell. At the time of inoculation the medium was changed to Eagle’s MEM with 0-5% FCS.

**Peptides.** The derived amino acid sequence of RSV strain A2 P protein (Galinski, 1991) was used for simultaneous multiple solid-phase peptide synthesis according to the previously described method (Houghten, 1985). Peptide amides were synthesized by using t-Boc amino acids (Bachem) and p-methylbenzhydrylamine resin (Fluka) and cleaved by liquid hydrogen fluoride in a multi-vessel apparatus. The...
performed as follows. Different concentrations (from 0.1 to 20 μg/ml) of the competitive peptide together with a standardized dilution of MAb (giving 50% of the maximum A) in ELISA dilution buffer were mixed, added to the wells preadsorbed with either homologous or heterologous peptide and allowed to react for 3 h at 37 °C. Competition ELISA between the MABS and HCS was performed in a similar way using different dilutions (from 1:50 to 1:1000) of HCS as the competitive reactant. ELISA of peptide analogues, based on a double-antibody sandwich protocol, was performed as previously described (Joisson & Van Regenmortel, 1991), where affinity-purified MAB C771 (1 μg/ml) and rabbit anti-P49 sera (at a dilution of 1:1000) were used as the source of the first and capture antibodies, respectively. Results were calculated as the percentage of binding.

**Results**

**Reactivity of MABS with peptides representing the RSV P protein**

Twenty-three 20 amino acid long peptides (with a 10-residue overlap) that covered the complete amino acid sequence of the P protein of RSV (strain A2) were synthesized. These peptides were tested in an ELISA with 14 MABS raised against the P protein of RSV. Strong reactivities ($A_{492} > 1.0$) were demonstrated for the following MABS (Fig. 1): C771 with peptides no. 48 and 49 (further designated as $P_n$, where $n$ represents the peptide no., e.g. P48); RS203 with P49; RS205 with P56; M1624 and P62. The binding competition assay demonstrated that P49 provided a more favourable epitope composition and (or) conformation for MAb C771 recognition than P49 did (data not shown). The specific sequences for the reactive peptides are shown in Table 1.

**ELISA reactivity of paired and late convalescent human sera with selected peptides**

Each of seven paired human sera samples (from persons of different ages) was tested in ELISA based on using P49, P56 and P62 as antigens. According to these data (Fig. 2), the difference in reactivity values of acute and convalescent sera (at one given dilution) was greater with P56 than with P49 or P62. The reactivities of the RSV-positive HCS are summarized in Fig. 3 for P48, P49, P56 and P62. P49 and P48 demonstrated approximately the

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence*</th>
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<tbody>
<tr>
<td>P48</td>
<td>R_{48}KPLVSFKEDPSDNPESK_{100}</td>
</tr>
<tr>
<td>P49</td>
<td>P_{49}TPSNDNPEKLYKETIETF_{184}</td>
</tr>
<tr>
<td>P56</td>
<td>S_{56}ARDGIRDAMIGLREEMIFK_{168}</td>
</tr>
<tr>
<td>P62</td>
<td>E_{62}KLNNLLEGNDSDLITEDF_{241}</td>
</tr>
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* The bold amino acids represent identified antigenic determinants containing epitopes (underlined). The amino acid positions in the original RSV strain A2 P protein sequence (Galinski, 1991) are indicated.

![Fig. 2. ELISA reactivity of seven RSV IgG EIA-positive acute (marked ‘A’ at the top of the figure) and convalescent (‘C’) human sera with P49, P56 and P62 peptides. The $A_{492}$ value was calculated (as described in Fig. 1 legend) and expressed as percentiles (boxed), mean values and standard deviation on the same figure by use of the StatView SE + Graphics computer program (Macintosh).](image)

![Fig. 3. ELISA reactivity of 20 HCS (diluted 1:100) samples with selected P protein peptides. Results are calculated and expressed as in Fig. 2.](image)
same reactivity; 20 and 19 sera (with a $A_{492}$ cut-off value of 0.2) were positive, respectively. The reactivities of the sera with P62 were less pronounced (16 sera were positive). Only 12 sera were positive in the ELISA based on P56. The other P protein peptides demonstrated low reactivity (equal to or below the cut-off value) with the same sera (data not shown). The binding competition assay also revealed that antibodies in human sera could block each of the three antigenic sites (represented by P49, P56 and P62) that were identified by the respective MAbs (data not shown).

Characterization of epitopes with peptide analogues

To define the dependence on individual amino acids of epitopes recognized by different MAbs we synthesized substituted peptide analogues of P49, P56 and P62, in which each amino acid (starting from the C-terminal cysteine) of the original peptide sequences was substituted in turn by glycine. These analogues were tested by ELISA with MAbs C771, RS203, RS205 and M1624. As shown in Fig. 4(a), the stretch of amino acids Asn$_{96}$ to Tyr$_{102}$ together with Glu$_{94}$ and Glu$_{107}$ were crucial (i.e. reduced binding of 50% and more) for MAb C771 recognition of corresponding peptide analogues. Alternatively, the substitution of Pro$_{94}$, Pro$_{95}$, Asp$_{95}$, Thr$_{105}$ and Phe$_{109}$ increased C771 binding (to more than 100% compared with the original peptide) to the respective analogues. By way of contrast, MAb RS203, which reacts with the same antigenic site, was dependent on only four amino acids: Pro$_{92}$, Phe$_{98}$, Lys$_{100}$ and Leu$_{101}$ (Fig. 4b). Hence the antigenic site represented by P49 harboured at least two, and probably many more, distinct epitopes. The four amino acid stretch Ile$_{166}$ to Ala$_{169}$ as well as Asp$_{164}$, Ile$_{171}$ and Leu$_{178}$ were necessary for MAb RS205 recognition of the P56 epitope (Fig. 4c). The substitution of Ala$_{162}$ and Ile$_{178}$ caused MAb RS205 to bind more than 100% to the respective analogues. Within P62, only amino acids Asp$_{223}$, Leu$_{231}$ and Leu$_{238}$ were critical, whereas Glu$_{223}$ and Glu$_{229}$ were responsible for increased binding of MAb 1624 (Fig. 4d). In order to exclude conformational changes in the peptide analogues during adsorption on the solid phase we confirmed the results of some of these experiments by use of a double-antibody sandwich protocol (see details in Methods).

Anti-RSV reactivity of antibodies raised against P protein peptides

Rabbit anti-peptide sera raised against P49 (R203), P56 (R209), P62 (R204) and, as a control, the N-terminal (amino acids 1 to 20) P protein peptide (R202) were tested for reactivity in ELISA with both RSV (strain A2)-infected cells and the respective synthetic peptides. All sera contained high levels of anti-peptide and anti-virus antibodies (Table 2) except that the antiserum raised against P56 showed only an insignificant reactivity with whole cell-associated viral antigens. The anti-N-terminal peptide serum demonstrated a high level of antibodies that were directed against the whole cell-
-associated viral antigens. To confirm the specificity of these rabbit anti-peptide sera, RIPA using 35S-labelled A2-infected cells as antigen was performed (Fig. 5). Antisera R203 (Fig. 5, lane 13) and R204 (Fig. 5, lane 12) strongly precipitated 'P' (i.e. the mature fully phosphorylated form of the P protein, apparent Mr, 36K) together with 'Pbrk' (i.e. the N-terminally truncated form of the P protein, apparent Mr, 23K) (Caravokyri et al., 1992) and also gave a weak precipitation of the N protein (Mr, 42K) which was assumed to represent coprecipitated material. These two forms of the P protein were also precipitated by the P protein-specific MAbs C771 (Mufson et al., 1985) and RS205 (Waris, 1992) (Fig. 5, lane 9). We could not observe any of these P protein forms in the RIPA with R202 (Fig. 5, lane 14) and R209 (Fig. 5, lane 11), whereas unidentified protein bands (with apparent Mr, 39K and 100K) together with a slightly increased (in comparison with control values) amount of the N protein in the R209 precipitate was observed. In other experiments (data not shown), a protein band of Mr, 34K (which may be the mature unphosphorylated form of the P protein; Caravokyri et al., 1992) appeared. Coprecipi-

tation of the N protein together with cellular protein (probably actin) was identified by RIPA of the RSV antigen using pooled preimmune sera from the same rabbits (Fig. 5, lane 10).

Discussion

Using a large collection of P protein-specific MAbs (Mufson et al., 1985; Orvell et al., 1987; Waris, 1992) in an assay of the antigenicity of P-specific peptides, three antigenic regions were identified. Screening these peptides by using 20 RSV-positive HCS confirmed the preferential immunoreactivity of the three sites defined by the four MAb-reactive peptides P48, P49, P56 and P62. Tests with human sera did not indicate the occurrence of any additional dominant antigenic sites. Data obtained in a parallel study (Garcia et al., 1993) demonstrated that the large antigenic area covered by enzyme-cleavage peptides is localized within N-terminal (amino acids 2 to 41), middle (91 to 211) and C-terminal (221 to 241) stretches of the RSV P protein sequence. The reactivity of the middle (91 to 211) and C-terminal (221 to 241) regions is supported by the present data. Predicted \( \alpha \)-helical loops and (or) \( \beta \)-turns combined with the simultaneous occurrence of high values for surface exposure were considered to be good indicators for potential antigenicity (Van Regenmortel, 1989). Our computer-derived data analysis (see Methods), based on the method of Kyte & Doolittle, showed the preferential existence of hydrophilic regions covering stretches Pro91 to Asp110 (P49), Ser141 to Lys180 (P56) and Glu221 to Phe241 (P62) (data not shown). The method of Chou & Fasman applied in the same computer predictions indicated an \( \alpha \)-helical structure for Pro48 to Asp110 (P49), as well as sequential \( \alpha \)-helical and \( \beta \)-sheet structures for the Ser141 to Lys180 (P56) and Glu221 to Phe241 (P62) regions (data not shown). Thus, all of the identified epitopes correspond to regions that are indicated to be antigenic by various criteria used for the prediction of the both surface exposure and secondary structure of proteins.

Our ELISA data confirm earlier observations (McIntosh & Chanock, 1985; Collins, 1991) concerning the abundance of anti-P protein antibodies in HCS from RSV-infected persons and revealed the antibody response targets to lie within three conserved (Mallipeddi & Samal, 1992) regions of the P protein molecule. Moreover, ELISA competition data (data not shown) demonstrated that RSV-positive HCS contained antibodies directed against each of two antigenic determinants (represented by peptide antigens, i.e. P49 and P62) that were recognized by MAbs (i.e. C771 and M1624, respectively). At the same time, broader deviation in the ability of HCS to compete with the MAb...
for P49 (in comparison with P62, data not shown) reactivity suggested a particular variation in the HCS epitope specificity within these determinants. The amino acid dependence (Fig. 4a, b, d) of the P49 and P62 determinants was markedly different. Since the P49 antigenic determinant consists of two (or more) epitopes, and the genetic restriction should work at the level of the epitopes (Norrby et al., 1989), there is a potential for each individual to respond to at least one of these in genetically outbred human populations. Such observations, of course, should be confirmed by using a larger panel of HCS. On the other hand, the competition phenomenon shown between MAbs and HCS may be useful for the design of a site-directed serological assay. ELISA based on each of P49, P56 and P62 or a combination thereof (data not shown) should be used further to examine whether acute and convalescent phases of RSV infection can be detected, as well as to monitor the efficiency of RSV vaccination. Another approach based on the labelled peptides could be used for the same purposes. Peptide substituted analogues may also be used as negative controls in these tests.

The ELISA of anti-peptide rabbit sera (Table 2) showed a high immunogenicity of four particular P protein peptides, i.e. the N-terminal amino acids 1 to 20, P49, P56 and P62. At the same time, the ELISA (Table 2) and RIPA (Fig. 5) cross-reactivities of these sera with parental viral antigen were different. The high ELISA titre of anti-N-terminal peptide serum (R202) suggested that the corresponding P protein site was available in RSV-infected cell-associated material, but that this was not enough to achieve a quantitative P protein recognition among the soluble RSV-infected cell proteins in RIPA (Fig. 5, lane 14). The low anti-virus ELISA titre of anti-P56 sera (R209) was well correlated with the absence of RIPA cross-reactivity (Fig. 5, lane 11) indicating that P56 could elicit antibodies that are not cross-reactive with either whole cell-associated or soluble parental viral proteins. Hence, in terms of peptide immunochromy (Van Regenmortel & Neurath, 1990) both the N-terminal peptide and P56 may be discontinuous antigenic determinants, whereas P49 and P62 probably represent continuous ones.

Specific MAbs, polyclonal anti-peptide sera and peptides mimicking determinants of the proteins may be quite useful tools for structural–functional research, as others have observed for VSV (Yamashita & Kawai, 1990) or HIV (Neurath et al., 1992). Our anti-peptide animal sera (R203 and R204) were able both to detect RSV-infected cells and to precipitate two different forms specifically (i.e. 'P' and 'Pbrk') of P protein (Caravokyri & Pringle, 1992) (Fig. 5, lanes 13 and 12). The Pbrk forms, i.e. N-terminally truncated P protein (possibly by cellular proteases), which contains all three antigenic determinants identified, was present for a short time in RSV-infected cells (Caravokyri et al., 1992; our unpublished data). In some RIPA experiments (data not shown) using R204 serum a third form, Pint (probably a product of internal initiation from the P gene open reading frame and subsequent translation, and which should contain P56 and P62 determinants), was identified. This was labelled and very rapidly disappeared during a 10 min chase in the late phase of infection, as others observed earlier (Caravokyri et al., 1992). The exact role of these Pbrk and Pint forms in RSV infection is presently unclear. The highly acidic C-terminal stretch (last 10 amino acids) of P, Pbrk and Pint contains the third antigenic determinant (represented by P62) and, by analogy to phosphoproteins of other viruses, may be a polymerase attachment site (Banerjee & Barik, 1992) or a site of functional role in RSV pathogenesis, as well as their involvement in protein–protein interactions within the replicative complex, are now being investigated.

In conclusion, our present data agree with previous observations (Dyberg & Oldstone, 1986; Norrby et al., 1989; Appel et al., 1990) suggesting that polar hydrophilic (asparagine and serine) and hydrophobic (lysine) amino acids together with non-polar hydrophilic (proline) and hydrophobic (phenylalanine, leucine and isoleucine) amino acids occur most frequently in the B cell epitope composition. Taking into account the fact that some of the amino acids that are critical for antibody–peptide interaction were localized outside of the epitopes, we report here the existence of three antigenic determinants within the Pro4 to Asp116 (P49), Ser141 to Lys186 (P56) and Glu221 to Phe241 (P62) stretches of the RSV P protein sequence. The determinants contained the mapped immunodominant epitopes, whose functional role in RSV pathogenesis, as well as their involvement in protein–protein interactions within the replicative complex, are now being investigated.

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References


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