Mapping of epitopes and structural analysis of antigenic sites in the nucleoprotein of rabies virus

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Linear epitopes on the rabies virus nucleoprotein (N) recognized by six MAbs raised against antigenic sites I (MAbs 6-4, 12-2 and 13-27) and IV (MAbs 6-9, 7-12 and 8-1) were investigated. Based on our previous studies on sites I and IV, 24 consecutively overlapping octapeptides and N- and C-terminal-deleted mutant N proteins were prepared. Results showed that all three site I epitopes studied and two site IV epitopes (for MAbs 8-1 and 6-9) mapped to aa 358–367, and that the other site IV epitope of MAb 7-12 mapped to aa 375–383. Tests using chimeric and truncated proteins showed that MAb 8-1 also requires the N-terminal sequence of the N protein to recognize its binding region more efficiently. Immunofluorescence studies demonstrated that all three site I-specific MAbs and one site IV-specific MAb (7-12) stained the N antigen that was diffusely distributed in the whole cytoplasm; the other two site IV-specific MAbs (6-9 and 8-1) detected only the N antigen in the cytoplasmic inclusion bodies (CIB). An antigenic site II-specific MAb (6-17) also detected CIB-associated N antigen alone. Furthermore, the level of diffuse N antigens decreased after treatment of infected cells with cycloheximide. These results suggest that epitopes at site I are expressed on the immature form of the N protein, but epitope structures of site IV MAbs 6-9 and 8-1 are created and/or exposed only after maturation of the N protein.

Introduction

Rabies virus belongs to the genus Lyssavirus of the family Rhabdoviridae. The rabies virion comprises five major virus proteins: the nucleoprotein (N), phosphoprotein (NS), the RNA polymerase protein (L), the surface-projecting glycoprotein (G) and the matrix protein (M). The N, NS and L proteins are associated with the genomic RNA of the virus to form a ribonucleocapsid (RNP) complex. The RNP complex is surrounded by a lipid bilayer associated with the G and M proteins.

The N protein is the major internal component of the rabies virion. It has been shown that the N protein has group-specific antigenic determinants that are shared by all rabies viruses and there are some antigens that are common to rabies and rabies-related viruses (Flamand et al., 1980). MAbs against these determinants have been used to define and discriminate between the antigenicity of rabies and rabies-related viruses. It has been reported recently from several laboratories that the rabies virus N protein alone can induce protective immunity against lethal infection in mice and dogs (Fekadu et al., 1992; Fu et al., 1991; Fujii et al., 1994; Lodmell et al., 1991). Furthermore, along with cytotoxic T lymphocytes, the antiviral activity of anti-N antibodies plays an important role in this protective mechanism (Lafon & Lafage, 1987; Lodmell et al., 1993; Takita-Sonoda et al., 1993). Accordingly, the nature and properties of the antigenic structures of the rabies virus N protein have become subjects of considerable interest.

Antigenic structures of the rabies virus N protein have been investigated by competitive binding assays with MAb panels. This kind of assay has allowed the discrimination of at least three or four antigenic sites on the N protein molecule, as reported for some strains such as ERA (Lafon & Wiktor, 1985) and RC-HL (Minamoto et al., 1994). Furthermore, use of...
peptide fragments generated by chemical or enzymatic cleavage and synthetic peptides has also been a useful means for epitope mapping as demonstrated for some epitopes composed of 10–25 aa located on the C-terminal region of the N protein (Dietzschold et al., 1987). We have shown that antigenic sites I and IV, and antigenic sites II and III on the N protein are composed of linear- and conformation-dependent epitopes, respectively (Minamoto et al., 1994). Moreover, by using deletion mutants expressed in E. coli (Goto et al., 1995), we showed that the epitopes of antigenic sites I and IV mapped to a region composed of 24 aa residues located in the C-terminal part of the N protein.

In this study, we have tried to define more precisely the epitope structure of sites I and IV of the N protein using a set of overlapping synthetic octapeptides covering aa 357–387 in the protein sequence, and using N- and C-terminal-deleted mutant N proteins.

Methods

● Virus and cells. The RC-HL strain of the rabies virus was used in this study. This strain is presently used in the production of cell culture vaccines for dogs in Japan (Ishikawa et al., 1989). The virus was propagated in baby hamster kidney (BHK-21) cells, which were grown in Eagle’s MEM supplemented with 10% tryptose phosphate broth (Difco), 5% calf serum and antibiotics.

● MAbs. The MAbs used in this study (Table 1) have been described previously (Minamoto et al., 1994). The epitopes of these MAbs mapped to a small region composed of aa 360–383 of the N protein (Goto et al., 1995). Additionally, antigenic site II-specific MAbs 11-6 and 6-17, corresponding to the conformational epitope on the N protein (Minamoto et al., 1994), and MAb 15-13, directed to a linear epitope on the glycoprotein of rabies virus (Luo et al., 1997), were also used in some experiments.

Table 1. MAbs used in this study

Data published by Minamoto et al. (1994). All of the MAbs used reacted with RC-HL-infected cells at a dilution of at least 1:100,000 by IFA.

<table>
<thead>
<tr>
<th>MAb</th>
<th>Antigenic site</th>
<th>Reactivity with indicated viruses*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Rabies</td>
</tr>
<tr>
<td>6-4</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>13-27</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>12-2</td>
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<td>+</td>
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<tr>
<td>6-9</td>
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<tr>
<td>7-12</td>
<td>IV</td>
<td>+</td>
</tr>
<tr>
<td>8-1</td>
<td>IV</td>
<td>+</td>
</tr>
</tbody>
</table>

* Rabies includes RC-HL, HEP-Flury, CVS and ERA strains of the virus. Duv, Duvenhage virus; Mok, Mokola virus; Lagos, Lagos bat virus.
+ No reactivity with HEP-Flury strain at 1:100 dilution.

Table 2. List of octapeptides used for epitope mapping

<table>
<thead>
<tr>
<th>Peptide no.*</th>
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<td>24 (380)</td>
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* The amino acid position in the native N protein corresponding to the first amino acid of each peptide is indicated in parentheses.

● Synthetic peptides. Twenty-four consecutive overlapping octapeptides (Table 2) were designed to mimic and cover the entire region of the 31 aa sequence (aa 357–387) of the RC-HL N protein. These octapeptides were synthesized by a solid-phase method on polyethylene pins using a commercial Fmoc kit (Epitope scanning kit; CHIRON MIMOTOPES).

● ELISA. Binding of MAbs to the synthetic peptides was analysed by ELISA as described by the manufacturer of the Epitope scanning kit. Colour was developed using O-phenylenediamine instead of 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfate), and quantified by determining the absorbance at 490 nm.

● Construction of N/366 and N/374 plasmids for expression in E. coli. A portion of the N gene, corresponding to aa sequences 212–366 or 212–374 of the RC-HL N protein, was amplified by PCR. The primers used were as follows: upstream primer, 5' GGCATATGTTTTCCTCCGGATT 3'; downstream N/366 primer, 5' AAGTTCTTCTCATCTCTGAAAGA 3'; and N/374 primer, 5' CAGTTCGCTGGCCTCGTATTTCT 3'. The DNA fragments obtained by PCR were digested with EcoRI and C-terminal coding fragments were ligated with N-terminal coding fragments obtained from the full-length N cDNA by EcoRI digestion (Fig. 2A). The nucleotide sequence of the region synthesized by PCR was confirmed by the dideoxy sequencing method. The N/366 and N/374 mutant proteins contained aa 1–366 and aa 1–374, respectively, of the N protein. The mutant N genes were subcloned into a bacterial plasmid expression vector, pET3a.
Expression of the mutant genes was induced in the same way as described previously (Goto et al., 1995). Other mutant N genes (N/359 and N/383) were constructed as described previously (Goto et al., 1995). Mutant proteins expressed in E. coli were subjected to SDS–PAGE (Laemmli, 1970) and immunoblot analysis (Minamoto et al., 1994).

Preparation of chimeric N proteins and C-terminal fragments. The common Styl restriction site in the N cDNA was used to generate chimeric N genes composed of fragments from the N cDNAs of RC-HL and the HEP-Flury strains. The cDNAs were digested with Styl and recombined to exchange the N-terminal and C-terminal fragments. The chimeric N genes were subcloned into the NdeI site of pET3a. An NdeI site (CATATG) was generated by the PCR method by introducing a point mutation at the codon of aa 318 (Tyr) of the RC-HL N protein. Digestion with NdeI yielded a C-terminal coding fragment that encoded aa 319 (Met) to 450. The C-terminal fragment of the RC-HL N cDNA was subcloned into the NdeI site of pET3a. The C-terminal coding fragment of HEP-Flury N cDNA was obtained by digestion with BglII and BamHI from the full-length N cDNA of the HEP-Flury strain inserted in pUC19. The BglII–BamHI fragment was subcloned into the BamHI site of pET3a. The resultant plasmid expressed a fusion protein composed of N-terminal 13 aa derived from the major capsid protein of T7 phage and a polypeptide composed of aa 324–450 of the HEP-Flury N protein. Expression of the chimeric N proteins and the C-terminal fragments was induced as described above.

Immunofluorescence assay (IFA). BHK-21 cells were infected with the RC-HL strain (m.o.i. of 5) and incubated for 48 h. Infected cells were fixed with a mixture of 50% acetone and 50% methanol for 10 min, then incubated with anti-N MAbs. The bound MAbs were visualized by immunostaining with FITC-conjugated second antibody raised against mouse IgG (ICN Immunobiologicals).

Treatment of infected cells with cycloheximide. At 48 h post-infection, cycloheximide was added to the culture medium of the infected BHK cells at a final concentration of 100 µg/ml. The cells were further incubated for 1 h and then fixed as described above. Detection of the N antigens was performed by IFA as described above.

Results

Identification and mapping using synthetic peptides of epitopes that constitute antigenic sites I and IV

The epitopes located in antigenic sites I and IV of the N protein were mapped to aa 360–383 (Goto et al., 1995). To define these epitopes more precisely, we designed and synthesized 24 overlapping octapeptides which covered the whole sequence of the presumed epitope region (Table 2). These peptides were tested for their binding ability with six site I- and site IV-specific MAbs. Site I-specific MAbs reacted specifically with one or two of the synthetic peptides (Fig. 1). Although MAb 6-4 displayed strong reactivity with most of the peptides at a high dilution of 1:10⁶, the strongest reactivity was observed with two peptides (nos 3 and 4). MAb 13-27 reacted with two peptides (nos 2 and 3), whereas MAb 12-2 reacted only with peptide no. 3. A conformational epitope-specific MAb raised against site II (MAb 11-6) and an anti-G MAb (MAb 15-13) gave negative results. From these results, we infer that the epitopes of MAbs 6-4, 13-27 and 12-2 map to aa 359–367, 358–366 and 359–366, respectively. This inference means that these three epitopes were located in a small region between aa 358 and 367 (RFFRDKEKLQ; Fig. 3).

Among the site IV-specific MAbs, only MAb 8-1 was clearly shown to react with peptide no. 3, whose sequence corresponded to aa 359–366. The other two MAbs (7-12 and 6-9) did not react with any of the peptides prepared.

Epitope mapping of the site IV-specific MAbs using deletion mutant proteins

Next, epitope mapping of some other site IV-specific MAbs (6-9 and 7-12) was performed using deletion mutants of the N protein because of a lack of a reaction with any of the synthetic peptides. Two deletion mutants (N/366 and N/374), which lacked the C-terminal sequence of the N protein after aa 366 and 374, respectively (Fig. 2A), were prepared. These mutants were expressed in E. coli and examined for their reactivity with MAbs by Western blotting. MAb 6-9 reacted with N/366 and N/374, but neither of these mutant proteins were recognized by MAb 7-12 (Fig. 2B). Although MAbs 6-9 and 7-12 were able to react with N/383 (Goto et al., 1995), which was composed of aa 1–383 of the N protein, they did not react with N/359, which lacks the amino acids after position 359 (Fig. 2B). This result was despite the binding of N/359 to guinea-pig anti-rabies virus polyclonal antibodies. Based on these reactivity profiles, we infer that the epitopes of MAbs 6-9 and 7-12 are located in separate regions, i.e. the regions composed of aa 360–366 and 375–383. MAb 8-1 reacted with both mutants, a result consistent with the inference mentioned above that the epitope of MAb 8-1 mapped to aa 359–366. These results also indicate that two independent epitopes of site IV are contained in a small region, extending 25 aa from 359 to 383 (Fig. 3).

Identification of a collaborating N-terminal domain that is required for the efficient binding of N protein to MAb 8-1

MAb 8-1 failed to recognize the N antigen of the HEP-Flury strain, but it recognized that of other strains including RC-HL, ERA, PV and SAD B19 (Table 1). Consequently, we compared the amino acid sequence of the N protein among these strains (Goto et al., 1994; Anzai et al., 1997) and found that a mutation at position 13 is unique in the HEP-Flury strain; glutamine at this position is replaced by arginine. To examine whether this substitution can affect the reactivity of MAb 8-1, chimeric genes were constructed by recombining the restriction fragments, the N-terminal fragments (aa 1–42) and the C-terminal fragments (aa 43–450) of N cDNA of the RC-HL and HEP-Flury strains (Fig. 4A). Chimeric N genes were expressed in E. coli and tested for their binding to MAb 8-1 by Western blot assays (Fig. 4B). As already described in our previous study (Goto et al., 1995), MAb 8-1 reacted weakly with the HEP-Flury N protein (Fig. 4B, lane 2). The RC-HL/HEP chimeric N protein reacted well with MAb 8-1 (Fig. 4B, lane 4). In contrast, the reactivity of the chimeric N
Fig. 1. Reactivity of consecutive overlapping octapeptides with MAbs. Reactivity of peptides with MAbs was determined by ELISA and is shown as the absorbance at 490 nm. The numbering of peptides corresponds to that given in Table 2. MAbs and their dilution were as follows: MAb 6-4, 1:10^6; MAb 13-27, 1:10^4; MAb 12-2, 1:10^4; MAb 8-1, 1:10^4; MAb 7-12, 1:10^4; MAb 6-9, 1:10^4; MAb 11-6 (specific for site II), 1:10^4; and MAb 15-13 (raised against a rabies virus glycoprotein), 1:10^3.

Fig. 2. Mapping of antigenic site IV by Western blot assay. (A) Schematic diagram of the structure of mutant proteins expressed in E. coli. (B) Western blot assay of mutant proteins. Mutant proteins were expressed in E. coli. Total bacterial proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Binding of MAbs to mutant proteins was detected with horseradish peroxidase-conjugated antibodies against IgG and with an ECL detection system (Amersham).

protein HEP/RC-HL was much reduced (Fig. 4B, b, lane 3). Regardless of similarity to the core sequence of the MAb 8-1 epitope, MAb 12-2 displayed strong reactivity with both of the chimeric N proteins (Fig. 4B, a). The reactivity of each chimeric N protein with MAbs 6-4, 13-27, 6-9 and 7-12 was much the same as that with MAb 12-2 (data not shown).

To confirm the effect of amino acid substitution at position 13, we further examined by Western blot assays the reactivity...
Epitope mapping of rabies virus N protein

Fig. 3. Alignment of the sequences from aa 358–387 of N proteins. RC-HL (Goto et al., 1994), ERA (Ertl et al., 1989), PV (Tordo et al., 1986) and SAD B19 (Conzelmann et al., 1990) strains have no substitution in this region. The amino acid sequences of the HEP-Flury (HEP) and CVS strains were determined by A. Kawai (unpublished data) and Mannen et al. (1991), respectively. Those of Duvenhage (Duv), Mokola (Mok) and Lagos bat (Lagos) viruses were reported by Bourhy et al. (1993). Dashes indicate amino acid identity. The open box indicates the antigenic domain of site I and the hatched boxes indicate the antigenic domains that form antigenic site IV.

Fig. 4. Reactivity of chimeric N proteins with MAbs. The N-terminal region of aa 1–42 and the C-terminal region of aa 43–450 of the N protein of RC-HL and HEP-Flury strains were exchanged. Chimeric N proteins were expressed in E. coli and tested for their reactivities with MAbs by Western blot assay. (A) Structures of chimeric N proteins. The StyI site was used for recombination of the N cDNA between the RC-HL and the HEP-Flury strains. (B) Reactivity of chimeric N proteins with MAbs. MAbs 12-2 (a) and 8-1 (b) were used at 10^4- and 10^3-fold dilutions, respectively. Lanes: 1, the full-length N protein of strain RC-HL (GN10); 2, the full-length N protein of the HEP-Flury strain; 3, the chimeric N protein composed of the N-terminal region (aa 1–42) of the N protein of the HEP-Flury strain and the C-terminal region (aa 43–450) of the N protein of strain RC-HL; and 4, the chimeric N protein composed of the N-terminal region (aa 1–42) of the N protein of strain RC-HL and the C-terminal region (aa 43–450) of the N protein of strain RC-HL.

of MAb 8-1 with the C-terminal fragments of RC-HL and HEP-Flury N proteins that included the core sequence of the presumed epitope region (Fig. 5). MAb 8-1 displayed weaker reactivity than MAb 12-2 with the C-terminal fragments of both strains (Fig. 5 A, B, lanes 3 and 4). In this experiment, MAb 8-1 did not display as great a difference in the reactivity with the C-terminal fragments (Fig. 5 B, lanes 3 and 4) as that seen with the full-length N proteins of the RC-HL and HEP-Flury strains (Fig. 4 B, lanes 1 and 2). In contrast, MAb 8-1 displayed strong reactivity with an internal deletion mutant (GN10S), which comprised the N-terminal 42 aa and C-terminal 105 aa of the RC-HL N protein (Fig. 5 B, lane 2). On the other hand, as we reported previously, MAb 8-1 did not react at all with the C-terminal-deleted mutants of the RC-HL N protein, which lacked the presumed epitope region (Goto et al., 1995). These results indicate that, in addition to the core sequence of the epitope (aa 359–366), the N-terminal region of the N protein is also necessary for MAb 8-1 to react with the N protein efficiently. Furthermore, the amino acid substitution at position 13 of the N protein would affect its binding ability to MAb 8-1, as seen in the HEP-Flury strain which lacked this ability.

Different IFA staining patterns displayed by site I-specific and site IV-specific MAbs in infected BHK-21 cells

Although two of the three epitope sequences in the site IV region are also present in a small region of antigenic site I, site I-specific and site IV-specific MAbs did not compete with each other in the competitive binding assays (Minamoto et al., 1994). To explain this discrepancy, we postulated that the site I and site IV regions might be expressed as different forms of the N protein. This interpretation was examined by IFA, in which we compared the intracellular localization of N antigens detected by these MAbs.

IFA staining patterns indicated that site I-specific MAb 13-27 detected N antigen (Fig. 6), which was distributed diffusely through the entire cytoplasm. N antigens in the cytoplasmic inclusion bodies (CIB) were also recognized by these MAbs. A site IV-specific MAb (7-12) gave the same staining pattern seen with the site I-specific MAbs, whereas the remaining site IV-specific MAbs (6-9 and 8-1) mainly detected the CIB-associated N antigen. These observations suggest that there are at least two forms of N protein in the cell, and that the
diffusely distributed N proteins were recognized by both site I-specific MAbs and MAb 7-12, whereas the site IV epitopes detected by MAbs 6-9 and 8-1 were only exposed on the N proteins incorporated into CIBs.

To investigate the relationship between the diffusely distributed and CIB-associated N protein, we performed IFA with conformational epitope-specific MAb 6-17. MAbs specific for site II or site III detected only the CIB-associated N antigen and not the diffuse N antigen (Fig. 6).

Finally, we investigated the effects on the intracellular distribution of N protein antigens after the blockade of N protein synthesis with cycloheximide. IFA demonstrated that, after a 60 min treatment with cycloheximide following 48 h infection, the intracellular distribution of N protein antigen was greatly altered (Fig. 6). The amount of diffuse N antigen detected by MAb 13-27 was much reduced, whereas the amount of CIB-associated N antigen increased (Fig. 6) compared with results obtained in the absence of cycloheximide treatment. Similar observations were also obtained with other site I-specific MAbs (6-4 and 12-2; data not shown). In contrast, the distribution of the CIB-associated N antigens which were recognized by MAbs 6-9 and 8-1 and conformational epitope-specific MAb (6-17) was not altered regardless of cycloheximide treatment (Fig. 6). These results suggest that the diffuse type of N protein may represent the immature form of the N protein, on which site IV epitopes are not exposed, whereas such epitopes may be created, exposed, or both after the maturation of N protein as detected on the CIB-associated N proteins.

Discussion

In this study, we have investigated fine structures of the linear epitopes that constitute antigenic sites I and IV on the rabies virus N protein. Based on the reactivity patterns of MAbs with a set of synthetic octapeptides, three site I epitopes mapped to the same small region composed of aa 358–367. Mapping of site IV could be achieved by using these synthetic peptides and the C-terminal-deleted N proteins N/359, N/366, N/374 and N/383. Linear epitopes of site IV were mapped to two independent separate regions, which were composed of aa 359–366 for epitopes of MAbs 6-9 and 8-1, and aa 375–383 for the epitope of MAb 7-12. Three of the five epitopes of sites I and IV, which are composed of aa 358–367, are also shared by rabies-related viruses (Table 1). In other words, amino acids in this region are highly conserved and constitute common antigenic sites of the lyssaviruses as detected by MAbs 6-4, 12-2 and 6-9.

Fig. 5. Reactivity of the C-terminal fragment of N protein with MAbs. Full-length N protein and three different fragments were expressed in E. coli and tested for their reactivities with MAbs 12-2 (A) and 8-1 (B) by Western blot assay. Lanes: 1, the full-length N protein of strain RC-HL (GN10); 2, the internally deleted fragment of RC-HL N protein (GN10S); 3, the C-terminal fragment aa 319–450 of strain RC-HL; and 4, the C-terminal fragment aa 324–450 of strain HEP-Flury (this fragment was longer than the C-terminal fragment of RC-HL strain because 13 aa derived from the major capsid protein of T7 phage were included at the N terminus of the HEP-Flury N fragment). The reactivity of each deleted N protein recognized by MAb 12-2 was also obtained with MAbs 6-4, 13-27, 6-9 and 7-12.

Fig. 6. Changes in the distribution of N protein in the cytoplasm of rabies virus-infected cells. BHK-21 cells were infected with the RC-HL strain (m.o.i. of 5) and 48 h later, cells were treated with cycloheximide (100 µg/ml) for 1 h. N protein was detected by MAbs and bound MAbs were visualized with FITC-conjugated antibodies raised against mouse IgG. The staining pattern of diffusely distributed N protein recognized by MAb 13-27 was also obtained with MAbs 6-4, 12-2 and 7-12. The staining pattern of CIB-associated N protein recognized by MAb 8-1 was also obtained with MAb 6-9. MAb 6-17 recognized the conformationally determined epitope.

Discussion

In this study, we have investigated fine structures of the linear epitopes that constitute antigenic sites I and IV on the rabies virus N protein. Based on the reactivity patterns of MAbs with a set of synthetic octapeptides, three site I epitopes mapped to the same small region composed of aa 358–367. Mapping of site IV could be achieved by using these synthetic peptides and the C-terminal-deleted N proteins N/359, N/366, N/374 and N/383. Linear epitopes of site IV were mapped to two independent separate regions, which were composed of aa 359–366 for epitopes of MAbs 6-9 and 8-1, and aa 375–383 for the epitope of MAb 7-12. Three of the five epitopes of sites I and IV, which are composed of aa 358–367, are also shared by rabies-related viruses (Table 1). In other words, amino acids in this region are highly conserved and constitute common antigenic sites of the lyssaviruses as detected by MAbs 6-4, 12-2 and 6-9.
By using the oligopeptides obtained by chemical and enzymatic cleavages of the ERA virus N protein, Dietzschold et al. (1987) mapped the epitope of their site I-specific MAb (377-7) to a region composed of aa 374–383. Since the epitope of our site IV-specific MAb (7-12) mapped to a similar region to that of MAb 377-7, both MAbs may recognize related structures. The precise epitope structures in these studies, however, may not be identical, because MAb 7-12 reacted strongly with the N protein of rabies virus strain CVS, whereas MAb 377-7 did not react. Substitution of Thr by Ser at position 377 of the protein may have been responsible for the loss of the reactivity of MAb 377-7 with the protein (Dietzschold et al., 1987; Rupprecht et al., 1991).

In this study, we showed that a small region composed of eight amino acids (aa 359–366) is shared by the two independent antigenic sites I and IV of the N protein, but the MAbs raised against these sites did not compete with each other for binding to the protein (Minamoto et al., 1994). From these considerations, we think that the antigenic sites I and IV may be expressed on different forms of the N protein. Consistent with this assumption, two types of rabies virus N protein were recognized in the IFA based on their different patterns of distribution in the cell. These were the diffuse and the CIB-associated N proteins. The diffuse type is recognized by the site I-specific MAbs, whereas the site IV-specific MAbs recognize mainly the CIB-associated N antigen. An exceptional case, however, was site IV-specific MAb 7-12; this MAb recognized the diffuse N protein but did not compete against site I-specific MAbs. This discrepancy may be explained by separation of the site I epitopes from the MAb 7-12 epitope on the N protein.

The CIB-associated N protein, but not the diffuse N protein, was recognized by the conformational epitope-specific MAbs. Cycloheximide treatment reduced the amount of diffuse N protein in the cell. Accordingly, it seems likely that the diffuse type of N protein may not be fully folded and may be rich in linear structures. In contrast, the CIB-associated N protein may be a mature form having gained its own native structures, exposing on its surface the conformation-dependent epitopes. A similar phenomenon has also been reported for another negative-stranded virus. Arnheiter et al. (1985) detected two forms of N protein in cells infected with vesicular stomatitis virus (VSV) using IFA with two different MAbs. The CIB-associated rabies virus N protein may correspond to the nucleocapsid-associated VSV N protein, because the rabies virus-specific CIB is thought to be composed of viral nucleocapsids (Hummeler et al., 1968). Recently, structures of the paramyxovirus N protein have also been investigated using MAbs as sensitive probes (Buckland et al., 1989; Deshpande & Portner, 1984; Gill et al., 1988; Ryan et al., 1993). Hirano et al. (1992) observed that the antigenicity of the free measles virus N protein was different from that of the nucleocapsid-associated N protein in the cell. Also, Gombart et al. (1993) noted that the measles virus N protein was initially synthesized as a relatively unfolded form and underwent conformational changes to assume a more folded mature form. Further studies will be required to fully explain the lack of reactivities of some MAbs with different conformations of the N protein.

Minamoto et al. (1994) described the antigenic site IV of the rabies virus as being composed of at least six epitopes, three of which were found to be linear, the others being conformational. Taken together, we think that aa 359–383 are not only involved in constituting the linear epitopes of sites I and IV, but that they are also included in the conformational epitopes of site IV. Furthermore, the structures of the site IV epitopes seem to be more complicated than we expected.

The first problem involves MAb 8-1. The epitope structure of site IV-specific MAb 8-1 is conserved in most rabies virus strains (Fig. 3). However, the HEP-Flury N protein displayed different reactivity with MAb 8-1 as seen in the IFA and Western blot assays. This difference may be caused by secondary structures or conformational changes of the protein. There are two possible mechanisms: first, the epitope was masked owing to an HEP-Flury-specific conformation; second, some change occurred in a region of the N protein that gave the antibody access to the epitope site (Westhof et al., 1984; Davies et al., 1988). Masking can be excluded, because the amino acid sequences of the N protein are highly conserved among rabies virus strains (Kissi et al., 1995). This is especially true for amino acid residues like Cys, Gly and Pro; changes in these residues are considered to affect protein conformation.

MAb 8-1 displayed strong reactivity with the mature form of the RC-HL N protein, whereas it reacted only weakly with the C-terminal fragment of the protein. This differential reactivity indicates that the N-terminal domain of the N protein is required for efficient binding of MAb 8-1 to the epitope site in the C-terminal region. MAb 8-1 may be reactive when the N-terminal domains are situated in the neighbourhood of the epitope site after the maturation of the N protein. From these assumptions, the most plausible explanation for the unique defectiveness of HEP-Flury N protein would be as follows: replacement at position 13 of the uncharged Glu residue by a positively charged Arg residue might cause minor structural changes around the epitope site, which would affect the access of the MAb to the epitope site. Similarly, Hwang & Lai (1993) reported that the reactivity of a MAb to the hepatitis delta antigen was lost upon alteration of the local conformation, regardless of the existence of the epitope sequence. In further studies, X-ray crystallographic analysis of the N protein will be indispensable to confirm our interpretation.

The second problem with the site IV-specific MAbs is that, although the site IV-specific MAbs 6-9 and 7-12 recognized the linear epitopes on the N protein, they displayed no reactivity with any of the peptides which were expected to contain an epitope-mimicking structure. These MAbs may require a longer peptide than octapeptides and they may bind to the more complex structures that would be created in
collaboration with the flanking sequences. As noted above, IFA indicated that the epitope of MAb 6-9 seems to be created and exposed, or both, after N protein maturation.

The data presented in this paper should provide a useful basis to probe the fine structure of rabies virus N protein.

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