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Antigenic Structure of Rabies Virus Glycoprotein: Ordering and Immunological Characterization of the Large CNBr Cleavage Fragments

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After rabies virus glycoprotein was treated with CNBr, the peptide mixture was fractionated by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. CNBr-cleaved peptide fragments were resolved into seven peptide bands under reducing conditions and six peptide bands under nonreducing conditions. The isolated nonreduced polypeptides were further analyzed by electrophoresis under reducing conditions. The N-terminal amino acid sequences were determined for the peptides in each of the isolated bands. The sequence data identified eight CNBr peptides and allowed the peptide fragments to be ordered within the deduced amino acid sequence of the glycoprotein. Analysis of the nonreduced CNBr peptides revealed two conformations of the glycoprotein. Two CNBr peptide fragments were specifically immunoprecipitated with a hyperimmune anti-rabies glycoprotein serum. These two and one other CNBr peptide induced the production of rabies virus-neutralizing antibodies, indicating the existence of at least three distinct antigenic sites on the rabies virus glycoprotein.

Rabies virus glycoprotein is the only rabies virus antigen that induces production of and reacts with virus-neutralizing (VN) antibodies (6). Monoclonal VN antibodies directed against the glycoprotein, however, have revealed a great diversity among rabies virus strains (9). Such antigenic differences among the glycoproteins most likely reflect differences in the amino acid sequence of the glycoprotein molecule. For definition of the antigenic structure and variability of the rabies virus glycoprotein a detailed analysis of the glycoprotein structure is necessary. Recently, the nucleotide sequence of the cDNA cloned from the rabies virus glycoprotein mRNA was determined (1). The reading frame was identified by partial amino acid sequence analyses of the glycoprotein amino and carboxy termini (15). For locating the amino acid sequences of the rabies virus glycoprotein that represent the structural basis of antigenic variability it is necessary to identify the regions of the molecule that induce VN antibodies. Fragmentation of proteins is an effective method for determining the immunogenic structure of protein antigens (2, 12, 13, 22). In this report, we describe our investigation of the ability of peptide fragments derived from the rabies virus glycoprotein by cyanogen bromide (CNBr) cleavage to induce rabies VN antibodies. N-terminal amino acid sequence analysis, which allowed the ordering of the CNBr fragments within the glycoprotein amino acid sequence, and the immunological characterization of these CNBr fragments enabled us to map the immunogenic regions of the rabies virus glycoprotein.

MATERIALS AND METHODS

Preparation of virus and glycoprotein. The ERA strain of rabies virus was propagated on BHK-21 cell monolayers and purified as previously described (24). As the tracer for protein and peptide purification, ERA virus labeled with [^{14}C]lysine and [^{14}C]leucine was prepared as previously described (8). The glycoprotein was purified as recently described (15).

CNBr cleavage of rabies virus glycoprotein. A total of 2.5 mg of purified glycoprotein labeled with [^{14}C]lysine and [^{14}C]leucine was precipitated with 4 volumes of ethanol. The precipitate was dried and dissolved in 70% formic acid. After 100 mg of CNBr was added, the solution was kept in the dark at room temperature for 24 h to allow complete cleavage at the carboxyl site of the methionine residues. Reagents were then removed by repeated evaporation and water resuspension. The remaining peptides were dissolved in 0.05 M Tris-hydrochloride containing 2% sodium dodecyl sulfate (SDS) and precipitated with 8 volumes of ethanol.

Purification and analysis of CNBr peptides by SDS-PAGE. Electrophoresis was performed in a 15 to 20% gradient SDS-polyacrylamide slab gel with a 5% (wt/

vol) stacking gel as described by Laemmli (14). For resolving reduced peptides, a CNBr-cleaved peptide mixture prepared from 1.5 mg of ^{14}C-labeled protein containing 300,000 cpm was dissolved in 250 μl of water containing 1% SDS and 5% 2-mercaptoethanol. For preparing nonreduced peptides, purified glycoprotein was treated for 2 h at 37°C with 0.1 M iodoacetamide in 1 M Tris-hydrochloride, pH 8.1. The protein was precipitated with ethanol and then treated with CNBr as described above, and the CNBr fragments were dissolved in 1% SDS–0.01 M iodoacetamide.

The peptide mixture was incubated for 5 min in a boiling water bath and then applied to the gel. After polyacrylamide gel electrophoresis (PAGE) had been performed at constant voltage (100 V), the gel was dried without prior fixation and exposed to X-ray film for 7 days at −70°C. The developed film was then superimposed on the gel, and the individual peptide bands were cut out and eluted with 0.05 M NH_4HCO_3–0.5% SDS. Some of the bands were visualized by staining the gel with ethidium bromide (23).

For analysis of purification, ^{14}C-labeled CNBr peptides containing 1,000 to 3,000 cpm were electrophoresed in a 15 to 20% gradient gel, and the peptide bands were visualized by fluorography (3).

Sequence analysis. The eluted peptides were lyophilized, dissolved in a small volume of distilled water, and precipitated with 8 volumes of ethanol. Ten nanomoles of each peptide was coupled with 2-amino-1,5-naphthalenedisulfonic acid to the C-terminal residue by water-soluble carbodiimide as previously described (10). The N-terminal sequence of the CNBr peptides was determined by automated Edman degradation in a Beckman 890 C sequencer with a 1 M Quadrol program (no. 122974). The following modifications were used: (i) all drying steps were done at low cup speed to insure drying of the sample in the undercut; (ii) heptafluorobutyric acid was delivered at high cup speed to insure complete mixing with the sample; (iii) cleavage reaction time was increased over several steps, and the cup speed was varied between low and high; and (iv) SDS was added to the Quadrol (0.2% [wt/vol]) and to the sample (2.0 mg) (11). The thiozolinones were converted with a Sequemat P-6 Auto-Converter, and the phenylhydantoin amino acids were identified by high-performance liquid chromatography on an Ultrasphere ODS C-18 reverse-phase column (Altex) with an acetate buffer-acetonitrile gradient (21).

Amino acid analysis. Peptides were hydrolyzed for 24 h with 6 N HCl in evacuated tubes. The amino acids were derivatized with *O*-phthalaldehyde, and the derivatives were subsequently analyzed by reverse-phase chromatography as previously described (16).

Immunoprecipitation. CNBr peptide fragments labeled with [^3H]methionine were dissolved in 1% SDS, precipitated with ethanol as previously described (19), redissolved in 0.5 ml of 0.05 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl–1% Triton X-100–0.5% deoxycholate, and incubated with 5 μl of either rabbit antiserum against ERA glycoprotein or normal rabbit serum at 4°C for 15 h. The antigen-antibody complexes were precipitated by adding 50 μl of a 10% suspension of *Staphylococcus aureus* as previously described (19). The CNBr peptide fragments of ERA glycoprotein and the immunoprecipitable fragments were solubilized with a small volume of 1% SDS–1% 2-mercaptoethanol and electrophoresed in a 15 to 20% gradient polyacrylamide slab gel. The peptide bands were visualized by fluorography.

Immunization of mice. Mice were immunized with CNBr peptides of ERA glycoprotein purified by SDS-PAGE as described above. The major portion of SDS was removed from the peptide extract by precipitating the eluted peptides with ethanol and redissolving them in 0.05 M Tris-hydrochloride, pH 7.6. Six-week-old ICR mice were injected subcutaneously with 50 μg of peptide emulsified in complete Freund adjuvant. Mice were boosted on days 21 and 28 with 20 μg of peptide and incomplete Freund adjuvant and bled on day 35.

Virus neutralization test. The levels of VN antibody in the immunized mice were evaluated by the rapid fluorescence focus inhibition test as described elsewhere (20). Serial threefold dilutions of serum were reacted with 10^4 PFU of ERA virus for 60 min at 37°C. The non-neutralized virus was detected on BHK-21 cells after a 20-h incubation by staining the cells with fluorescein-conjugated anti-nucleocapsid antibody. The reciprocal of the highest serum dilution capable of reducing the number of infected cells by 50% was taken as the neutralization titer.

Radioimmunoassay. Purified UV-inactivated ERA virus, native glycoprotein, or SDS-denatured glycoprotein was used as the immunoadsorbent for radioimmunoassay. The assay was performed as previously described (9). Antigen (100 ng) was dried on soft plastic 96-well microtiter plates (disposable polyvinyl, U-shaped wells; Dynatech Laboratories). Plates were then washed with phosphate-buffered saline containing 10% agamma horse serum (GIBCO Laboratories). Threefold serial dilutions of serum were added in duplicate to the wells (25 μl per well), and the plates were incubated for 1 h at 37°C and then washed three times with phosphate-buffered saline. Each well then received 25 μl of ^{125}I-labeled goat anti-mouse antibodies (30,000 cpm; specific activity, 0.5 mCi/mg). Plates were again incubated at 37°C for 1 h and washed three times in phosphate-buffered saline. The bottoms of the wells were cut out with an incandescent wire, and radioactivity was determined in a gamma counter. The reciprocal of the highest dilution of serum binding 10% of the ^{125}I counts was taken as the endpoint titer.

Complement-dependent immunolysis. A total of 5×10^6 NA cells (4) were infected (or mock infected) with 25 PFU of ERA virus per cell for 1 h at 37°C. These cells were incubated for 16 h to allow expression of viral surface antigens, then trypsinized, and resuspended in 0.2 ml of medium containing 100 μCi of sodium [^{51}Cr]chromate. After 1 h at 37°C, the cells were washed twice and used as targets.

Dilutions of serum were incubated with target cells (5,000 per well) and guinea pig complement (Cappel; 5% final concentration) in V-bottom microtiter plates in a total volume of 100 μl. After 2 h at 37°C, the plates were centrifuged, and the supernatants were sampled for released ^{51}Cr. Specific ^{51}Cr release was calculated as a percentage of the maximum ^{51}Cr released by 0.1% cetrimide after subtracting the spontaneous release in the medium. Complement or antiserum alone did not affect the spontaneous release values. Titers are expressed as the reciprocal of the highest dilution giving 10% specific release. The endpoint of 10% of specific cell lysis was determined to be greater than the statistical variation of spontaneous release in replicate samples.

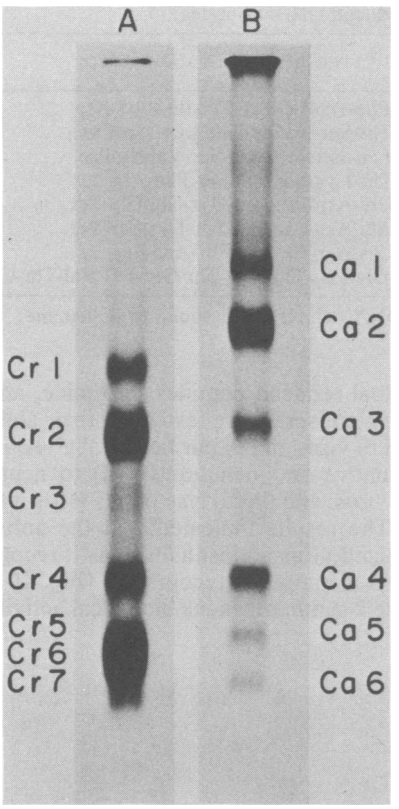

FIG. 1. Fluorography of CNBr peptide fragments labeled with [^{14}C]lysine and [^{14}C]leucine. Fragments were produced by CNBr cleavage of purified glycoprotein as described in the text. Fragments were solubilized with 1% SDS and reduced with 5% 2-mercaptoethanol (A) or alkylated with 0.1 M iodoacetamide (B) and resolved on a 15 to 20% polyacrylamide gel. The polyacrylamide gel was dried, and the fragments were visualized by fluorography (3).

RESULTS

Like the intact rabies virus glycoprotein, CNBr cleavage products of the glycoprotein are extremely insoluble in aqueous solutions. Even in 80% formic acid or 10 M urea it has not been possible to completely dissociate the peptides. Alkaline buffers (pH 7.5 to 8.0) containing 1% SDS can be used to solubilize the CNBr peptides of rabies glycoprotein, but peptides treated with SDS are difficult to separate by column chromatography. Thus, separation was performed by SDS-PAGE by the method of Laemmli, as the peptides did not elute from phosphate-urea gels.

CNBr peptide fragments labeled with [^{14}C]lysine and [^{14}C]leucine were separated in a 15 to 20% polyacrylamide gel (Fig. 1). Seven bands, designated Cr1 to Cr7 (for CNBr-reduced peptides), were resolved when the fragments were reduced before electrophoresis (Fig. 1A). Radiolabeled Cr3 was detected as a faint band, but ethidium bromide staining provided easy identification of this peptide. Cr5, Cr6, and Cr7 were also easily identified as separate bands with this staining method. In addition, peptides Cr1, Cr2, and Cr3 could be labeled with [^{3}H]glucosamine (data not shown), indicating that these three peptides contain the carbohydrate chains described by chemical analysis (7) and predicted from the amino acid sequence of the glycoprotein (1). When the CNBr fragments were isolated under nonreducing conditions (Fig. 1B) they resolved into six bands, designated Ca1 to Ca6 (for alkylated).

For isolation and further analysis of these peptides, 1.5 mg of glycoprotein labeled with [^{14}C]lysine and [^{14}C]leucine was treated with CNBr and separated by electrophoresis. About 70% of the radioactivity was recovered in the isolated peptides, which were then electrophoresed again (Fig. 2). Fragment Cr4-1 represents a subfraction. Each of the isolated peptides migrated as a single band.

N-terminal sequencing of reduced peptides. To map the CNBr peptides within the amino acid sequence of the glycoprotein deduced from the glycoprotein gene cDNA sequence, we determined the N-terminal sequence of each peptide.

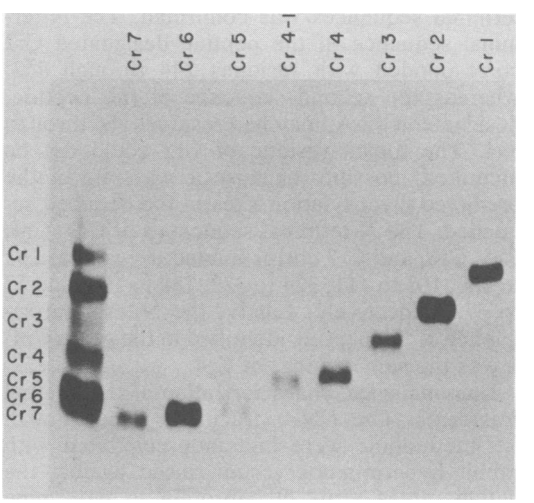

FIG. 2. Re-electrophoresis of reduced CNBr peptides. CNBr peptide fragments obtained from purified glycoprotein labeled with [^{14}C]lysine and [^{14}C]leucine were reduced with 2-mercaptoethanol and separated on a 15 to 20% polyacrylamide gel. The peptides were recovered from the gel as described in the text. A portion of each peptide was dissolved in 1% SDS–5% 2-mercaptoethanol and re-electrophoresed under the same conditions. The bands were visualized by fluorography.

TABLE 1. N-terminal sequences of reduced CNBr peptides

Peptide no.	N-terminal amino acid sequence
Cr1	H_2N-Lys-Phe-Pro-Ile-Tyr-Thr-Ile-Pro[a]-Asp. . .
Cr2	H_2N-Gln-Thr-Ser- -Glu-Thr-Lys-Trp-Cys. . .
Cr2-A	H_2N-Glu-Leu-Leu-Glu-Ser-Ser-Val-Ile-Pro. . .
Cr3	H_2N-Thr-Thr-Lys-Ser-Val-Ser-Phe-Arg. . .
Cr4	H_2N-Ala-Gly-Asp-Pro-Arg-Tyr-Glu-Glu-Ser. . .
Cr5	H_2N-Glu-Ala-Asp-Ala-His-Tyr-Lys-Ser-Val. . .
Cr6	H_2N-Ser-Cys-Asp-Ile-Phe-Thr-Asn-Ser. . .
Cr7	H_2N-Asn-Gly-Phe-Thr-Cys-Thr-Gly-Val-Val-Thr-Glu. . .

[a] This residue does not correspond to the sequence of the cDNA, where it was found to be leucine.

After being coupled with 2-amino-1,5-naphthalenedisulfonic acid, the peptides were subjected to automated Edman degradation. Derivatization with 2-amino-1,5-naphthalenedisulfonic acid and the addition of 0.2% SDS to the 1 M Quadrol reagent were necessary to insure degradation of the peptides. Table 1 lists the N-terminal sequences determined for the reduced peptides. The first seven residues from the N-terminus of peptide Cr1 were found to match the N-terminal sequence of the intact glycoprotein. However, the eighth residue, proline, differed from the deduced sequence. At each degradation step of fragment Cr2 two distinct residues were identified, indicating that this band consisted of two peptides. After analysis and comparison with the deduced amino acid sequence of the glycoprotein, the presence of two different N-terminal sequences was confirmed. The N-terminal sequence of the peptide designated Cr2 corresponded with residues 244 through 252, whereas the second sequence of the peptide, designated Cr2-A, matched residues 386 through 394. The fourth residue of Cr2 could not be identified, possibly because it was one of the predicted glycosylation sites in the deduced sequence. The N-terminal sequences of Cr3, Cr4, Cr5, Cr6, and Cr7 corresponded to residues 292 to 300, 103 to 111, 324 to 332, 188 to 195, and 57 to 67, respectively. Lastly, the N-terminal sequence of the peptide identified in the gel as Cr4-1 was the same as that of Cr4.

Immunological characterization of the isolated fragments. The CNBr fragments labeled with [^{3}H]methionine were immunoprecipitated with rabbit hyperimmune serum raised against the native rabies virus glycoprotein to determine whether anti-rabies antibodies could recognize any of these fragments. Cr3 and Cr4 were specifically immunoprecipitated (Fig. 3). The faint band (Fig. 3C) shows a small amount of Cr4 immunoprecipitated by normal rabbit serum. However, this represents less than 5% of the Cr4 precipitated by the anti-glycoprotein antiserum. To determine whether the purified CNBr peptide fragments are immunogenic, we injected individual reduced peptides into mice, and the resulting antisera were tested for their ability (i) to bind to virus and to purified viral glycoprotein (both native and denatured); (ii) to neutralize rabies virus; and (iii) to lyse rabies virus-infected cells. The results indicated that the antiserum recognized virus almost as well as it recognized native or denatured glycoprotein (Fig. 4).

Table 2 summarizes the biological activities of

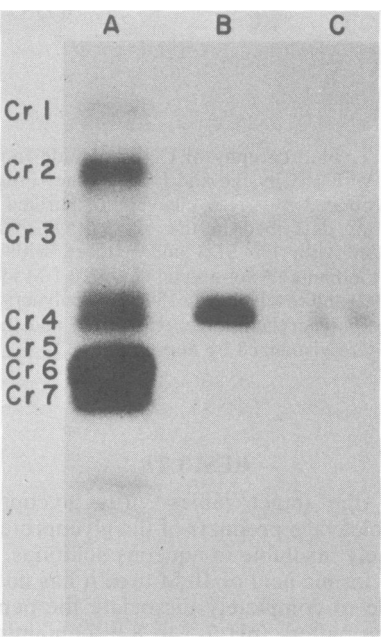

FIG. 3. Immunoprecipitation of ERA glycoprotein CNBr peptides with anti-rabies glycoprotein hyperimmune serum. CNBr peptides labeled with [^{3}H]methionine were dissolved in 0.5 ml of 0.05 M Tris-hydrochloride (pH 7.4)–0.1 M NaCl–1% Triton X-100–0.5% deoxycholate and incubated with 5 μl of rabbit antiserum directed against ERA glycoprotein (B) or normal rabbit serum (C). The antigen-antibody complexes were precipitated with a 10% suspension of *S. aureus* and resolved on a 15 to 20% polyacrylamide gel. (A) Peptide mixtures of CNBr-cleaved glycoprotein. Peptide bands were visualized by fluorography.

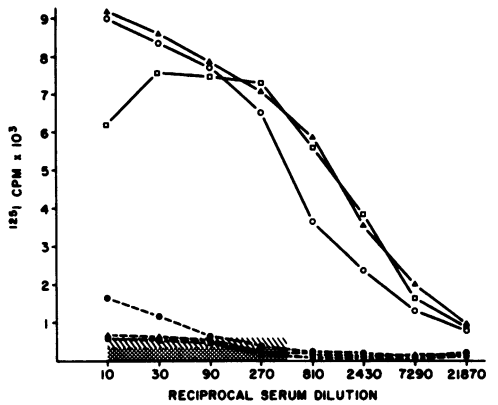

FIG. 4. Radioimmunoassay of antiserum raised against CNBr peptide Cr4 reactive with virus and with native and denatured glycoprotein. A 100-ng amount of virus (○), native glycoprotein (△), or SDS-denatured glycoprotein (□) was dried on soft plastic plates. Threefold serial dilutions of sera (- - - -, normal serum: ——, anti-Cr4 serum) were added in duplicate to wells. After the plates were washed extensively, the amount of bound antiserum was determined by adding ^{125}I-labeled goat anti-mouse antibodies. Dotted area, Complement-dependent lysis titer; cross-hatched area, VN activity.

TABLE 2. Serological characterization of antisera raised against native glycoprotein and CNBr fragments

Antiserum against:	Radioimmunoassay titer[a]	Neutralizing titer[b]	Complement-dependent lysis titer[c]
Cr1	21,870	180	180
Cr2 + Cr2-A	21,870	60	60
Cr3	21,870	270	180
Cr4	21,870	510	510
Cr5	7,290	0	0
Cr6	2,430	0	0
Cr7	810	0	0
Native glycoprotein	6,400	9,000	2,000

[a] The reciprocal of the highest dilution of serum binding 10% of the ^{125}I counts was taken as the endpoint.

[b] The reciprocal of the highest serum dilution capable of reducing the number of infected cells by 50% was taken as the neutralization titer.

[c] Titers are expressed as the reciprocal of the highest dilution giving 10% specific release.

the individual antisera. All of the peptides induced binding antibodies, although the titers obtained for Cr6 and Cr7 were significantly lower than those for the other peptides. In addition, antibodies induced by Cr1, Cr3, and Cr4 exhibited VN activity, with titers of 180, 270, and 510, respectively. These titers correlated well with the complement-dependent lysis titers. For the peptide mixture Cr2 + Cr2-A, neutralizing and complement-dependent lysis titers were found to be low. However, we could not determine which of the two peptides accounted for this activity or whether both did, since they could not be separated and tested individually. The use of peptides not coupled to high-molecular-weight carriers might account for the low VN activity of these CNBr peptide antisera. Antiserum raised against native rabies glycoprotein served as a control.

Intramolecular interaction of CNBr fragments. To study the possible interactions between the individual peptides in the intact molecule, we isolated CNBr peptide fragments under nonreducing conditions and analyzed them. For this purpose, the native nonreduced glycoprotein was carboxymethylated with iodoacetamide. Fragments Ca1 through Ca6 (Fig. 1B) were isolated under nonreducing conditions (see above). Re-electrophoresis of these peptides revealed that each again migrated as a single band (Fig. 5). When these same alkylated peptides were reduced and subjected to re-electrophoresis, peptides Ca1 and Ca2 were each resolved into two major bands (Fig. 6). After reduction,

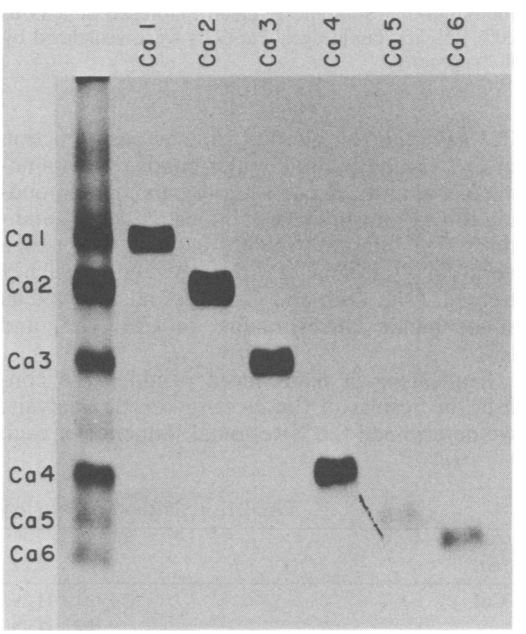

FIG. 5. Re-electrophoresis of nonreduced CNBr peptides. CNBr fragments obtained from purified glycoprotein labeled with [^{14}C]lysine and [^{14}C]leucine were treated with 0.1 M iodoacetamide and separated on a 15 to 20% polyacrylamide gel. Peptides were recovered as described in the text, and the isolated peptides were dissolved in 0.1 M iodoacetamide–1% SDS and re-electrophoresed under the same conditions. The bands were visualized by fluorography.

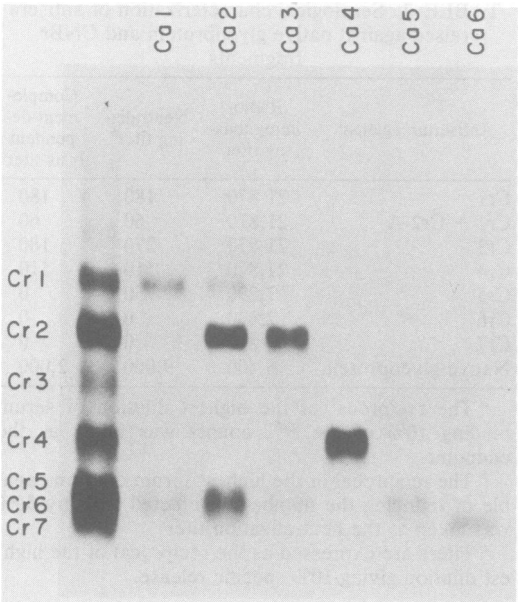

FIG. 6. Reduction and re-electrophoresis of CNBr peptides isolated under nonreducing conditions. CNBr peptides were isolated under nonreducing conditions as described in the legend to Fig. 5, and each of the isolated peptides was treated with 5% 2-mercaptoethanol–1% SDS and re-electrophoresed on a 15 to 20% polyacrylamide gel. Peptides were visualized by fluorography.

Ca1 gave rise to Cr1 and Cr6, whereas reduction of Ca2 resulted in two major bands corresponding to Cr2 and Cr6 and a minor band corresponding to Cr1. Furthermore, under reducing conditions Ca3 migrated mainly as Cr2, although a very small amount of Cr6 was seen. Similarly treated, Ca4, Ca5, and Ca6 were all resolved as single bands corresponding to Cr4, Cr5, and Cr7.

Sequencing of nonreduced peptides. To confirm the results of the electrophoretic analysis, we determined the N-terminal sequence of each alkylated peptide isolated under nonreducing conditions (Table 3). At each degradation step of Ca1, two quantitatively equivalent residues were obtained. The resulting N-terminal sequences of this peptide fragment matched the sequences of both the Cr1 and Cr6 peptides. N-terminal sequence analysis of Ca2 resulted in three residues per degradation step. Two of the residues were found in equal amounts, and a third was found in a significantly lower quantity. The two major peptide sequences corresponded with those of Cr2 and Cr6, whereas the minor peptide sequence matched the Cr1 sequence. Each degradation step of Ca3 also yielded a major and minor peptide identical to the N-terminal sequences of Cr2-A and Cr2, respectively. Single N-terminal sequences were obtained from peptides Ca4, Ca5, and Ca6 which corresponded to the sequences of Cr4, Cr5, and Cr7, respectively.

Both the electrophoretic analysis and the sequencing data for the nonreduced peptides strongly indicated that peptide Cr6 in its entirety was covalently linked by disulfide bridges either to peptide Cr1 or to peptide Cr2. However, part of each peptide Cr1 and Cr2 remained unlinked by disulfide bridges. The disulfide bridges are schematically illustrated in Fig. 7.

DISCUSSION

As an approach to localizing and defining the antigenic determinants of the rabies virus glycoprotein, we performed an immunological and chemical characterization of the peptide fragments isolated after CNBr cleavage of the glycoprotein. Fragmentation and chemical modification of protein antigens have been successfully employed in determining the location of antigenic determinants in many non-viral proteins (2) and viral antigens (12, 13, 22).

N-terminal sequence analysis and total amino acid analysis of each peptide showed that all methionine residues were converted into homoserine, indicating complete CNBr cleavage. This

TABLE 3. N-terminal sequences of nonreduced CNBr peptides

Peptide no.		N-terminal amino acid sequence[a]
Ca1	(a)	H_2N-Lys-Phe-Pro-Ile-Tyr-Thr-Ile-Pro-Asp-Lys-Leu. . .
	(b)	H_2N-Ser-Cys-Asp-Ile-Phe-Thr-Asn-Ser-Arg-Gly. . .
Ca2	(a)	H_2N-Gln-Thr-Ser- -Glu-Thr-Lys-Trp-Cys-Pro-Pro-Asp. . .
	(b)	H_2N-Ser-Cys-Asp-Ile-Phe-Thr-Asn-Ser-Arg-Gly-Lys. . .
	(c)	H_2N-Lys-Phe-Pro-Ile-Tyr-Thr-Ile-Pro. . .
Ca3	(a)	H_2N-Glu-Leu-Leu-Glu-Ser-Ser-Val-Ile-Pro-Leu-Val-His. . .
	(b)	H_2N-Gln-Thr-Ser- -Glu-Thr-Lys. . .
Ca4		H_2N-Ala-Gly-Asp-Pro-Arg-Tyr-Glu-Glu-Ser-Leu-His. . .
Ca5		H_2N-Glu-Ala-Asp-Ala-His-Tyr-Lys-Ser-Val. . .
Ca6		H_2N-Asn-Gly-Phe-Thr-Cys-Thr-Gly-Val-Val. . .

[a] More than one residue resulted at each degradation step for peptides Ca1, Ca2, and Ca3 (see the text).

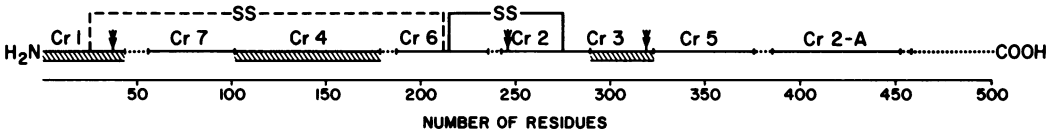

FIG. 7. Map of CNBr peptides and arrangement of the disulfide bridges. Peptides identified by their N-terminal sequence were ordered within the deduced amino acid sequence of the glycoprotein. Peptides that induced neutralizing antibodies are indicated (cross-hatched area). Double arrows indicate glycosylation sites. The arrangement of disulfide bridges was deduced from the reduction of nonreduced peptides as shown in Fig. 6 and from the sequencing data shown in Table 3.

allowed us to order the fragments within the primary amino acid sequence of the glycoprotein (Fig. 7). All of the large peptide fragments predicted from the deduced amino acid sequence were recovered, except for one fragment containing the C-terminal end of the glycoprotein. This was either lost during the isolation procedure or insoluble.

In an effort to determine the configuration of the intact glycoprotein, we investigated the intramolecular disulfide bridges. Analysis of CNBr peptides isolated under nonreducing conditions revealed that two nonreduced peptides, Ca1 and Ca2, analyzed under reducing conditions, each contained peptide Cr6 and either Cr1 or Cr2, respectively, indicating that Cr6 is linked by disulfide bonds to both Cr1 and Cr2. All of fragment Cr6 was bound to either Cr1 or Cr2 in the nonreduced form, whereas a portion of the Cr1 and Cr2 peptide fragments were unlinked. These findings suggest that the rabies glycoprotein exists in two different conformations. In one form, in which peptide Cr6 is linked to Cr1, a large loop containing at least 154 amino acid residues is formed. Note that the most immunologically active fragment (Cr4) was located within this loop. In the second conformation, in which Cr6 is linked to Cr2, a smaller loop is formed. The existence of two conformations may have significance for the assembly of the glycoprotein spike.

The existence of two electrophoretically distinct glycoprotein forms (GI and GII) has been established for certain rabies viruses (5, 8) and may reflect these two conformations. In the CVS strain, for example, both glycoprotein forms have identical amino acid structures, as judged by comparative tryptic peptide analysis, and differ only in their carbohydrate content (8). Since there is evidence that the structure of a protein can influence glycosylation (17, 18), we believe that GI and GII, which always appear in a ratio of 2:1, may represent two configuration forms of the same primary structure.

As shown by electrophoresis and N-terminal sequencing, all of the peptides in individual bands obtained under reducing conditions, except Cr2, represent homogeneous fragments. Immunization experiments with the purified reduced CNBr peptides showed that all of the fragments were able to induce antibodies that bound to rabies virus or to the viral glycoprotein. However, only two peptides reacted with anti-glycoprotein antiserum, and three peptides induced significant titers of VN antibodies, indicating the existence of three epitopes to which the VN antibodies bound. As compared with the antisera raised against native glycoprotein, the titers of antisera raised against these peptides were significantly lower. Thus, the secondary or tertiary structure, which might be lost during isolation of the peptides, probably contributes greatly to the antigenic site. This is in agreement with the observations that VN monoclonal antibodies do not bind to CNBr fragments and that after mild reduction the glycoprotein loses 95% of its immunological activity (unpublished data). The strong correlation between antibody titers for complement-dependent lysis and the results in virus neutralization tests suggests that both processes are controlled by the same determinants.

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LITERATURE CITED

1. **Anilionis, A., W. H. Wunner, and P. J. Curtis.** 1981. Structure of the glycoprotein gene in rabies virus. Nature (London) **294**:275–278.
2. **Atassi, M. Z., and A. L. Kazim.** 1978. First consequences of the determination of the entire antigenic structure of sperm whale myoglobin, p. 19–40. *In* M. Z. Atassi and A. B. Stavitsky (ed.), Immunology of proteins and peptides I, vol. 98. Plenum Press, New York.
3. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83–88.

4. **Clark, H. F.** 1980. Rabies serogroup viruses in neuroblastoma cells: propagation, "autointerference," and apparently random back-mutation of attenuated viruses to the virulent state. Infect. Immun. **27**:1012–1022.
5. **Coslett, G. D., B. P. Holloway, and J. F. Obijeski.** 1980. The structural proteins of rabies virus and evidence for their synthesis from separate monocistronic RNA species. J. Gen. Virol. **49**:161–180.
6. **Cox, J. H., B. Dietzschold, and L. G. Schneider.** 1977. Rabies virus glycoprotein. II. Biological and serological characterization. Infect. Immun. **16**:754–759.
7. **Dietzschold, B.** 1977. Oligosaccharides of the glycoprotein of rabies virus. J. Virol. **23**:286–293.
8. **Dietzschold, B., J. H. Cox, and L. G. Schneider.** 1979. Rabies virus strains: a comparison study by polypeptide analysis of vaccine strains with different pathogenic patterns. Virology **98**:63–75.
9. **Flamand, A., T. J. Wiktor, and H. Koprowski.** 1980. Use of hybridoma monoclonal antibodies in the detection of antigenic differences between rabies and rabies-related virus proteins. II. The glycoprotein. J. Gen. Virol. **48**:105–109.
10. **Fosler, J. A., E. Bruenger, C. L. Hu, K. Albertson, and C. Franzblau.** 1973. A new improved technique for automated sequencing of non-polar peptides. Biochem. Biophys. Res. Commun. **53**:70–74.
11. **Gerber, G. E., R. J. Anderegg, W. C. Herlihy, C. P. Gray, K. Biemann, and H. G. Khorana.** 1979. Partial primary structure of bacteriorhodopsin: sequencing methods for membrane proteins. Proc. Natl. Acad. Sci. U.S.A. **76**:227–231.
12. **Jackson, D. C., L. E. Brown, R. J. Russell, D. O. White, T. A. Dopheide, and C. W. Ward.** 1980. Antigenic and immunogenic properties of influenza virus hemagglutinin fragments, p. 309–320. *In* W. G. Laver and G. M. Air (ed.), Structure and variation in influenza virus. Elsevier/North-Holland, Inc., Amsterdam.
13. **Jackson, D. C., R. J. Russell, C. W. Ward, and T. A. Dopheide.** 1978. Antigenic determinants of influenza virus hemagglutinin. I. Cyanogen bromide peptides derived from A/MEMPHIS/72 hemagglutinin possess antigenic activity. Virology **89**:199–205.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
15. **Lai, C. Y., and B. Dietzschold.** 1981. Amino acid composition and terminal sequence analysis of the rabies virus glycoprotein: identification of the reading frame on the cDNA sequence. Biochem. Biophys. Res. Commun. **103**:536–542.
16. **Lindroth, P., and K. Mopper.** 1979. High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with *O*-phthaldialdehyde. Anal. Chem. **51**:1667–1673.
17. **Nakamura, K., and R. W. Compans.** 1979. Host cell- and virus strain-dependent differences in oligosaccharides of hemagglutinin glycoproteins of influenza A viruses. Virology **95**:8–23.
18. **Rosner, M. R., L. S. Grinna, and P. W. Robbins.** 1980. Differences in glycosylation patterns of closely related murine leukemia viruses. Proc. Natl. Acad. Sci. U.S.A. **77**:67–71.
19. **Shih, T. Y., M. O. Weeks, H. A. Young, and E. M. Scolnick.** 1979. p21 of Kirsten murine sarcoma virus is thermolabile in a viral mutant temperature sensitive for the maintenance of transformation. J. Virol. **31**:546–556.
20. **Smith, J. S., P. A. Jager, and G. M. Baer.** 1973. A rapid tissue culture test for determining rabies neutralizing antibodies, p. 354–357. *In* M. Kaplan and H. Koprowski (ed.), Laboratory techniques in rabies. WHO Monogr. Ser. 23.
21. **Somack, R.** 1980. Complete phenylthiohydantoin amino acid analysis by high-performance liquid chromatography on ultrasphere-octadecyltrimethyloxysilane. Anal. Biochem. **104**:464–468.
22. **Versteegen, R. J., and S. Oroszlan.** 1980. Effect of chemical modification and fragmentation on antigenic determinants of internal protein p30 and surface glycoprotein gp70 of type C retroviruses. J. Virol. **33**:983–992.
23. **Vincent, A., and K. Scherrer.** 1979. A rapid and sensitive method for detection of proteins in polyacrylamide SDS gels: staining with ethidium bromide. Mol. Biol. Rep. **5**:209–214.
24. **Wiktor, T. J., B. Dietzschold, R. N. Leamnson, and H. Koprowski.** 1977. Induction and biological properties of defective interfering particles of rabies virus. J. Virol. **21**:626–634.