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Major Receptor-Binding and Neutralization Determinants Are Located within the Same Domain of the Transmissible Gastroenteritis Virus (Coronavirus) Spike Protein

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The spike glycoprotein (S) of coronavirus, the major target for virus-neutralizing antibodies, is assumed to mediate the attachment of virions to the host cell. A 26-kilodalton fragment proteolytically cleaved from transmissible gastroenteritis virus (TGEV) S protein was previously shown to bear two adjacent antigenic sites, A and B, both defined by high-titer neutralizing antibodies. Recombinant baculoviruses expressing C-terminal truncations of the 26-kilodalton region were used to localize functionally important determinants in the S protein primary structure. Two overlapping 223- and 150-amino-acid-long products with serine 506 as a common N terminus expressed all of the site A and B epitopes and induced virus-binding antibodies. Coexpression of one of these truncated protein S derivatives with aminopeptidase N (APN), a cell surface molecule acting as a receptor for TGEV, led to the formation of a complex which could be immunoprecipitated by anti-S antibodies. These data provide evidence that major neutralization-mediating and receptor-binding determinants reside together within a domain of the S protein which behaves like an independent module. In spite of their ability to prevent S-APN interaction, the neutralizing antibodies appeared to recognize a preformed complex, thus indicating that antibody- and receptor-binding determinants should be essentially distinct. Together these findings bring new insight into the molecular mechanism of TGEV neutralization.

The coronaviruses are important pathogens inducing enteric, respiratory, and neurologic disorders in human and animals. They are enveloped viruses with a large (~30-kb), positive-sense RNA genome. The virions are constituted of a nucleocapsid protein and three or four envelope proteins: the membrane (M), spike (S), and hemagglutinin-esterase (HE) glycoproteins and a minor structural protein (sM). In addition, the genomic RNA codes for a fairly large polyprotein containing RNA polymerase motifs and for a few other, likely nonstructural proteins (1, 24, 36).

The S protein, which forms the distinctive surface projections of the virions, is a glycosylated, acylated polypeptide with a high molecular mass (170 kilodaltons [170K] to 220K). S is a typical class I membrane protein, with a membrane anchor near the C terminus (36). S protein undergoes a slow maturation and probably forms a complex three-dimensional structure (10, 39, 41). In several coronaviruses, the S protein is cleaved to yield two subunits of similar sizes, S1 (amino half) and S2 (carboxy half), thought to correspond to the globular and stalk regions, respectively, of the spike. A coiled-coil structure predicted in the S2 subunit is believed to hold the S monomers into a stable structure, which is proposed to be trimeric (4, 10).

Numerous studies have provided direct or indirect evidence for the involvement of S protein in inducing a protective immune response or in viral pathogenicity (3, 17, 18, 40). These functions may be associated with the pivotal role that S plays in the initial stage of infection, both in the attachment of the virus to the cell surface and in the induction of fusion between viral and cellular membranes (5, 36, 38). In this regard, substantial progress has recently been made with the identification of molecules used by coronaviruses for gaining entry into cells. Several glycoproteins of the murine carcinoembryonic antigen family have been reported to be functional receptors for the mouse hepatitis virus porcine enteric coronavirus (42, 44). The transmissible gastroenteritis virus (TGEV) and human respiratory coronavirus (HCV) 229E both utilize a membrane-bound metalloprotease, the aminopeptidase N (APN), as a specific receptor (8, 43). Furthermore, determinants of the carcinoembryonic antigen and APN molecules involved in binding of the virus have been mapped on their primary structures (6, 16). In contrast, the viral determinant(s) responsible for the recognition of the cell receptor is still undefined, even though it is generally acknowledged that the S protein mediates this function.

TGEV replicates in the differentiated enterocytes of the small intestine and causes a fatal diarrhea in newborn piglets (31). TGEV belongs to the same genetic subset as feline infectious peritonitis virus, canine coronavirus, porcine epidemic diarrhea virus, and human HCV 229E (14). This subset is characterized by the lack of an HE gene and by an uncleaved S protein. The TGEV S protein derives from a 1,447-amino-acid-long precursor polypeptide of which a 16-residue signal peptide is removed (34). S is the only protein that induces antibodies that neutralize in the absence of complement (21, 22, 25, 32). Investigations of its antigenic structure have led to the identification of four to five major groups of B-cell-dependent epitopes, all located in the S1 part of the polypeptide chain (2, 7, 12, 19). Most of these epitopes are conformation dependent and require core glycosylation to be expressed (11, 22). Other functional determinants have been tentatively localized on the basis of comparison of the S protein sequences of viruses with distinct biological phenotypes. Thus, the about 200-amino-acid N-terminal region which is missing in the S protein encoded by porcine respiratory coronavirus (PRCV)

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and human HCV 229E is a candidate to contain determinants involved in enteropathogenicity (15, 26). It has also been proposed that the S protein of TGEV contains two receptor-binding sites: one located in this polymorphic N-terminal region and contributing to the enteric tropism, and the other in a 60-amino-acid-long stretch upstream the antigenic site A, which is highly conserved among TGEV and PRCV strains (35).

Our earlier studies led to the identification of a proteolytic fragment generated by collagenase cleavage of antibody-protected S protein, called CO-26K, which expressed most of the TGEV neutralization epitopes (12). The main purpose of the present study was to analyze the functional properties of CO-26K-derived, baculovirus-expressed polypeptides. Our results led us to conclude that this domain is primarily involved in the recognition of the APN receptor. Data on the effects of neutralizing antibodies on the interaction between the S and APN proteins are also presented.

MATERIALS AND METHODS

Cells, viruses, and MAbs. Wild-type and recombinant *Autographa californica* nuclear polyhedrosis baculoviruses were isolated, grown, and assayed in confluent monolayers of *Spodoptera frugiperda* insect cells (clone Sf9) maintained in Hink's medium plus 10% fetal calf serum, using standard procedures (37). The characteristics of the murine immunoglobulin G (IgG) antibodies (MAbs) directed against TGEV S or porcine APN have been reported elsewhere (8, 25). The anti-TGEV S MAb 1AF10 (19) was provided by L. Enjuanes (CSIC-UAM Canto Blanco, Madrid, Spain).

Construction of transfer vectors. The baculovirus transfer vector pVL941 (28) has a unique *Bam*HI cloning site located immediately downstream of the baculovirus polyhedrin promoter, into which (i) an APN or (ii) truncated TGEV S gene was inserted. (i) Plasmid pVLAPN was constructed by excising a *Bam*HI-*Bgl*II fragment from pBAP2, which contains the entire APN cDNA coding sequence (8), and ligating it to *Bam*HI-cut pVL941. (ii) Mutagenic oligonucleotides and PCR techniques were used to engineer 5′ and 3′ restriction sites and stop signals immediately 3′ to the final codons of the TGEV S-coding sequences, as shown in Fig. 1. Plasmid pVLS1 was constructed as follows. A truncated S-coding sequence which contained the natural TGEV S initiation codon (nucleotide [nt] +1) and an additional TAA stop codon (nt +2459) was generated by PCR of pZG35 (21) by using two oligonucleotides carrying a *Bam*HI cleavage site, 5′AATTCGAGCTCG GATCCCAACACACCATG (primer 505) and 5′GGTTGCA CGGATCCATCTTAATGTGTGACG, as 5′ and 3′ amplimers, respectively. The amplification product was digested with *Bam*HI and ligated into similarly cleaved pVL941 vector. For the next constructs, an intermediate plasmid (pBSpeps), which contained the TGEV S signal peptide sequence, was generated. The corresponding fragment was amplified by PCR with the primers 505 and 5′ATTAGTCAATCTAGAACA AGG (3′ amplimer containing an *Xba*I restriction site). The amplification product was digested by *Bam*HI and *Xba*I, ligated into similarly cleaved pBS(SK−), and sequenced to confirm the presence of the correct signal peptide sequence. pVLS223 was constructed by a three-way ligation into the pVL941 cloning site with a *Bam*HI-*Xba*I signal peptide fragment and a TGEV *Xba*I-*Bam*HI S223 fragment (nt +1564 to +2232), both electroeluted from a 8% acrylamide gel. The S223 fragment was generated by PCR amplification of pZG35 with the following 5′ and 3′ primers: 5′GGATCTAGAGG AAGCTTTTACACACAT, which contained an *Xba*I restriction site and a short additional sequence coding for amino acids Ser-Arg-Gly and 5′CCCAACTATGGATCCTTAAAT AACAGCTGC, which introduced a TAA stop codon and a *Bam*HI restriction site. The three subsequent constructs, pVLS150, pVLS120, and pVLS88, were generated by PCR of pVLS223 with oligonucleotide 505 as a common 5′ primer and 3′ primers 5′GTGTGGATCCGACTAATCGTGCACAC, 5′ CCGGATCCTTGGTTCATGTACGGG, and 5′TGGATCCA AATCAGAAAGGAC, which contain stop codons (nt +2025, +1929, and +1830) and a *Bam*HI cloning site, as 3′ amplimers. The amplification products were digested by *Bam*HI and ligated into the pVL941 cloning site.

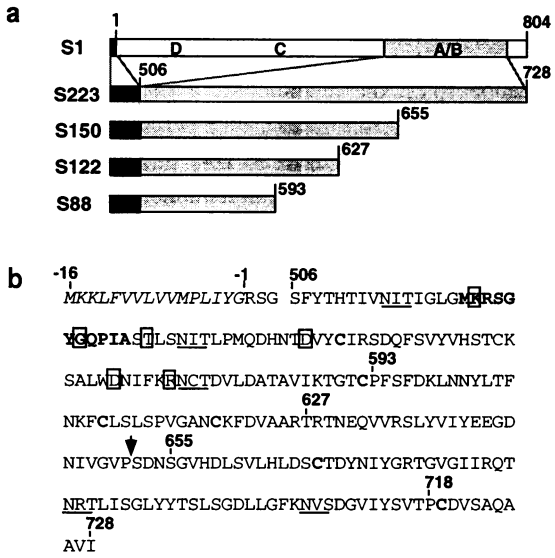

FIG. 1. Expression of various TGEV S-derived polypeptides in the baculovirus system. (a) Structures of the S1 polypeptide (amino-terminal half of S) and of four truncated derivatives, each designated by S followed by the number of amino acids. Numbers above the sequences indicate amino acid positions in the mature S polypeptide (34). The dark boxes represent the signal peptide. The relative positions of the antigenic sites A/B, C, and D mapped on S1 (12) are indicated. (b) Amino acid sequence of the S223 polypeptide. Three extraneous residues (RSG) were inserted at the junction between the signal peptide (in italics) and the S223 body sequence. The sequence of a synthetic peptide recognized by MAb 1AF10 (19) and Cys residues conserved among all viruses of the TGEV-related subset (15) are shown in boldface. Potential Asn-linked glycosylation sites are underlined. Boxed letters indicate the positions of amino acid substitutions identified in neutralization escape mutants (12, 19). The arrow indicates the new proposed C-terminal end of the collagenase 26K fragment (see text).

Recombinant baculoviruses. Two micrograms of each transfer plasmid was cotransfected into 1.5×10^5 Sf9 cells together with 200 ng of AcRP6-SC DNA linearized with the restriction enzyme *Bsu*36I (23), using the Lipofectin transfection procedure (Bethesda Research Laboratories). Six days after transfection, progeny virus was harvested and plaqued onto fresh Sf9 cells that were subsequently overlaid with 1.5% low-melting-point agarose (37). Five days later, 12 plaques arising from each transfection assay were excised and vortexed in Grace's medium to release virus. Recombinant viruses were screened by immunofluorescence on infected Sf9 cells and by immunoprecipitation of radiolabeled cell lysates and were then plaque purified and amplified as reported elsewhere (21). Resulting baculoviruses were designated AcAPN, AcS1, AcS223, and so forth.

Radiolabeling and immunoprecipitation. Monolayers of 2×10^5 Sf9 cells were infected or coinfected with baculoviruses at a multiplicity of infection of 3 to 10 PFU per cell. At 24 h postinfection (p.i.), the medium was replaced with Grace's medium plus 5% fetal bovine serum and incubated at 26°C for 20 h with 100 μCi of Tran^{35}S-label (approximately 70% L-methionine and 15% L-cysteine; ICN Biomedicals) per ml. The cells were lysed in 1 ml of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris [pH 7.8], 2% Triton X-100, 0.15 M NaCl, 0.6 M KCl, 0.5 mM $MgCl_2$, 10^3 Kallicrein units of aprotinin per ml). The resulting cell lysates were ultracentrifuged for 15 min at 12,000 rpm to remove cellular debris and stored at −70°C. For immunoprecipitation assays, aliquots of 0.05 to 0.2 ml of radiolabeled cell lysates were adjusted to 0.5 ml with RIPA buffer containing the appropriate MAb (5 μl of ascites fluid) and 40 μl of a 50% slurry protein A-Sepharose beads; after a 2-h incubation at room temperature under agitation, the immune complexes were extensively washed with RIPA buffer and then with 50 mM Tris buffer (pH 8)–0.5 M NaCl. The beads were heated for 3 min at 100°C in Laemmli sample buffer containing 5% 2-mercaptoethanol. The immunoprecipitated material thus released from the beads was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as indicated in the figure legends. Ascites fluids from murine hybridomas and from a feline infectious peritonitis virus-infected cat were used as sources of monoclonal and polyclonal antibodies, respectively (25).

Immunofluorescence, neutralization test, and ELISA. Baculovirus-infected Sf9 cells (multiplicity of infection of 10 PFU) were fixed at 2 days p.i. with ethanol-acetone and stained as described previously (21). Neutralization tests (500 PFU per well) and enzyme-linked immunosorbent assays (ELISAs) (500 ng of virions per well) for TGEV were performed by previously described procedures (8, 25).

BIAcore experiments. BIAcore (Pharmacia Biosensor, Uppsala, Sweden) allows analysis, in real time, of the kinetics and stoichiometry of the interactions between molecules trapped sequentially on a miniaturized biocaptor. The biocaptor consists of a hydrogel of dextran polymer and thus has properties very different from those of a membrane. The amounts of interacting ligands are monitored by surface plasmon resonance; the signal is expressed in resonance units (RU): a variation of 1,000 RU corresponds to a variation of immobilized or trapped material of 1 ng/mm^2. For all experiments, a flow rate of 5 μl/min was used in EDTA-free 0.15 M NaCl–*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.4 (HBS).

(i) MAb-binding interference map. Purified rabbit anti-mouse (RAM) IgG Fc immunoglobulins (RAMFc; Pharmacia) were first covalently linked to dextran chips (27). Twenty-five milliliters of a capture MAb (ascites fluid diluted 1/50 or 1/100) was injected, followed by 50 μl of a pool of 80 μg of normal mouse IgG per ml to block the unoccupied sites of the RAMFc. Then, 40 μl of S223 was injected as a crude supernatant of a 72-h-p.i. AcS223-infected Sf9 cells culture, followed by a second 50-μl injection of RAM-blocking immunoglobulin. The second MAb was injected for 5 min. Each of the tested MAbs was successively used as a capture MAb or as a second MAb. Nonspecific binding was monitored by replacing each specific MAb by the pool of RAM-blocking mouse IgG. RAM was regenerated by 15 μl of 1 M formic acid.

(ii) S1-APN interaction. After immobilization of RAMFc as described above, 25 μl of MAb 44.4 (capturing MAb for S1; ascites fluid diluted 1/50) was injected; 30 μl of a 1/5 dilution of S1 crude supernatant of infected Sf9 cells was then injected, followed by 40 μl of an APN urea extract (estimated APN concentration, 50 μg/ml) prepared as follows. Sf9 cells (10^8) were infected with AcAPN or control AcRP6-SC baculovirus (multiplicity of infection of 10 PFU). At 72 h p.i., cells were harvested by centrifugation at 800 × *g* for 10 min. The cell pellet was suspended in 4 ml of phosphate-buffered saline (PBS) and placed at −70°C. Five milliliters of frozen cells was thawed and suspended in 15 ml of 5 M urea (3 M urea in a 20-ml final volume). The mixture was incubated for 2 h in ice. The cell suspension was probe sonicated with four bursts, 1 min each at 50 W, with the tube immersed in an ice water bath. The lysate was centrifuged at 12,000 × *g* for 15 min to pellet insoluble material, and the supernatant was extensively dialyzed against PBS. The resulting material was kept at 4°C and used within 4 days after preparation.

Virus binding assay on ST cell membranes. Cell membranes prepared as previously described (29) were spotted onto a nitrocellulose sheet (5 μg of protein per spot; Schleicher & Schuell). Nonspecific binding sites were blocked with TBSA (3% bovine serum albumin, 20 mM Tris [pH 7.4], 150 mM NaCl). Purified labeled virions (2.5×10^4 cpm; 1×10^4 to 2×10^4 cpm/μg) prepared as described previously (11) were preincubated for 1 h at 37°C and 1 h at 4°C with antibodies (ascites fluid diluted 1/100) and then added to two spots of membranes. After 1 h at 37°C, the sheet was rinsed three times with TBSA–0.05% Tween 20, and radioactivity associated with each spot was measured in a scintillation counter.

Immunization experiments. Three-month-old BALB/c mice were injected intraperitoneally with 200 μl of a PBS suspension of 0.5×10^7 Sf9 cells infected by the AcS223 or AcS150 recombinant, emulsified in the same volume of complete Freund's adjuvant. One month later, the mice were boosted intraperitoneally with 10^7 cells in incomplete Freund's adjuvant. A third injection of 10^7 infected Sf9 cells without adjuvant was administered 1 month later, and retro-orbital blood samples were collected 15 days after the final injection.

RESULTS

Construction of baculoviruses expressing the TGEV S CO-26K domain and truncated derivatives. The N terminus of the CO-26K fragment was previously identified as serine 506 of the mature S polypeptide. While its C terminus was not determined experimentally, the CO-26K sequence was unlikely to extend beyond proline 718, as judged from both the estimated molecular mass of the collagenase-derived cleavage fragment and the substrate specificity of the enzyme (12). The first construct thus designed, which encoded the truncated form called S223, consisted of the 16-amino-acid-long S signal peptide (34) and the 223 S-specific amino acids from positions 506 to 728, joined together via a spacer of three extraneous residues (Fig. 1). This construct was subsequently used to derive three additional constructs encoding the amino-coterminal truncated forms S150, S122, and S88. Recombinant baculoviruses potentially expressing one of these sequences or the sequence called S1 (amino acid positions −16 to 804) were used in these experiments.

Immunoprecipitation analysis of the recombinant truncated forms of S. Cell lysate or supernatant from cultures infected with the different baculoviruses were first subjected to immunoprecipitation with a polyclonal antibody. S223-immunoprecipitated material essentially consisted of two bands (Fig. 2a) assumed to reflect different glycoforms, the lower one potentially bearing trimmed carbohydrates chains as previously established for baculovirus-expressed S protein (21). Five potential glycosylation sites in the S223 sequence may account for the discrepancy between the estimated (33 to 36K) and

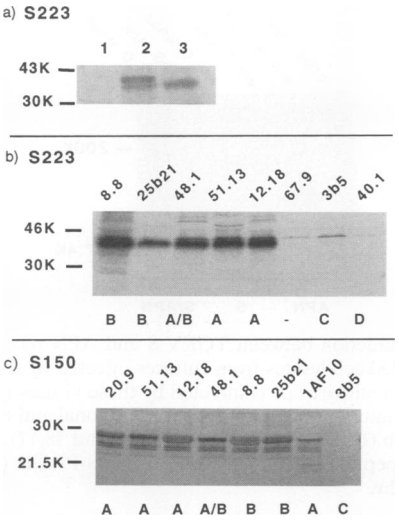

FIG. 2. Immunoprecipitation analysis of the S223 and S150 recombinant proteins. (a) Polypeptides immunoprecipitated by a polyclonal antibody from cell lysate of wild-type baculovirus-infected cells (lane 1), AcS223-infected cells (lane 2), or supernatant of AcS223-infected cells (lane 3) were resolved in an SDS–15% polyacrylamide gel and Coomassie blue stained. ^{35}S-labeled polypeptides immunoprecipitated by the indicated MAbs from AcS223 (b) or AcS150 (c) baculovirus-infected cultures were analyzed by SDS-PAGE (15% gel) and autoradiography. The letters beneath the lanes indicate the antigenic sites defined by the MAbs.

MAb	Site	S1	S223	S150	S122	S88
* 1AF10	A	■	■	■	▨	▨
* 51.13	A	■	■	■	□	□
* 12.18	A	■	■	■	□	□
* 20.9	A	■	■	■	□	□
* 48.1	A/B	■	■	■	□	□
* 8.8	B	■	■	■	▨	□
* 25b.21	B	■	■	■	▨	□
78.17	D	■	■	▨	□	□
* 40.1	D	■	□	□	□	□
5.2	D	■	□	□	□	□
69.21	D	■	□	□	□	□
* 3b.5	C	■	□	□	□	□
44.4	-	■	□	□	□	□

FIG. 3. Immunofluorescence analysis of the antigenicity of S-derived recombinant proteins. Baculovirus-infected cells expressing one of the five S derivatives were fixed, incubated with serial dilutions of the indicated MAbs, and processed for indirect immunofluorescence. Neutralizing MAbs (25) are marked with asterisks. The squares are filled according to the highest reciprocal dilution of antibody giving a clear positive signal: >12,500 (■); 2,500 ( ); 500 (▨); <100 (□).

predicted (25K) molecular masses. A substantial proportion of S223 protein, consisting of the lower-molecular-mass, potentially mature species, accumulated in the extracellular fraction of infected cultures (concentration estimated to be 10 μg/ml by reference to low-molecular-weight markers). This finding indicated that the protein was secreted, thus suggesting that the removal of the signal peptide was effective. Similar results were obtained with Ac150-infected cultures (not shown). The observed molecular mass (26 to 28K) of the S150 species (predicted molecular mass, 17K; three potential glycosylation sites) is more compatible with a cleavage of the virus-derived 26K collagenase fragment having occurred after proline 651 than after proline 718, as tentatively proposed earlier (12). Figure 2b shows that, like the CO-26K fragment, baculovirus-expressed S223 protein was efficiently recognized by several MAbs representative of the A or B antigenic site but not by MAbs to the C or D site or by MAb 67.9 (mainly restricted to the trimeric form of S [10]). The S150 protein exhibited an overall weaker reactivity (Fig. 2c), including toward MAb 1AF10, shown to define a site A linear epitope (19). Finally, no immunoreactive species could be observed in cultures infected with baculoviruses expressing the S122 and S88 proteins (predicted molecular masses of 14 and 10K, respectively).

Immunofluorescence reactivity pattern of the truncated S proteins. The antigenicity of each S recombinant derivative was further analyzed in an indirect immunofluorescence assay by using 13 MAbs known to exhibit closely similar reactivities toward baculovirus-expressed S and S1 (S amino half) proteins. From the data summarized in Fig. 3, several conclusions were drawn. (i) The S223 and S150 proteins were efficiently recognized by site A/B MAbs. (ii) The apparent reactivity of S223 was similar to that of S1 and higher overall than that of S150. (iii) S122 retained some reactivity toward MAb 1AF10 (linear epitope) and site B MAbs, whereas S88 was recognized by MAb 1AF10 only. (iv) MAb 78.17, which defines site D (mapped within the N-terminal first 212 S amino acids [12]), reacted with both S223 and S150. Altogether, the immunofluorescence data confirmed that S223 and S150 proteins expressed the whole set of A and B epitopes. Also, immunofluorescence allowed the detection of several epitopes expressed by these recombinant proteins (S223 with MAb 78.17, or S122 and S88 with MAb 1AF10) which could not be detected by immunoprecipitation.

Site A and B interference map in the BIAcore format. These antigenic sites were previously delineated on virion-associated S protein through competitive binding assays in a radioimmunoassay format. With the exception of MAb 48.1, assumed to define a common epitope, the MAbs defining one site did not show competitive binding toward the MAbs of the other site (7). It was of interest to determine whether such a topological relationship was maintained in the truncated recombinant proteins. To this end, a surface plasmon resonance analysis of S223-antibody interactions was performed with a BIAcore instrument. Pairs of MAbs belonging to separate sites showed an additive binding, whereas no additivity was observed with MAb 48.1 as a first antibody (Table 1). The site B MAb 8.8 behaved similarly to MAb 25b.21 (not shown). In addition, none of the MAbs showed self-additivity. This result, together with the stoichiometry of capture of S223 by the different MAbs (molar ratio ranging from 1 to 2.3 monomer equivalent by IgG mole; not shown), indicates that S223 was essentially in a monomeric state. Finally, in agreement with the immunofluorescence data, little if any capture of the S122 and S88 species

TABLE 1. S223 interference map with anti-site A and B MAbs in the BIAcore format

Capture MAb (site)	Binding of indicated 2nd MAb[a]				
	12.18	20.9	51.13	48.1	25b21
12.18 (A)	−47	−28	−43	−68	**200**
20.9 (A)	−60	−83	−67	−77	**268**
51.13 (A)	−79	−66	−126	−84	**311**
48.1 (A/B)	−77	−59	−70	−99	−66
25b.21 (B)	**74**	**122**	**200**	−109	−126

[a] Values correspond to RU binding levels of the second MAb after the following sequence: RAMFc; capture anti-A or -B MAb; S223 protein from cell lysate; blocking normal mouse IgG; second anti-A or -B MAb. Significant binding values are printed in boldface. Negative RU values reflect partial ligand dissociation occurring when a sequential injection is performed.

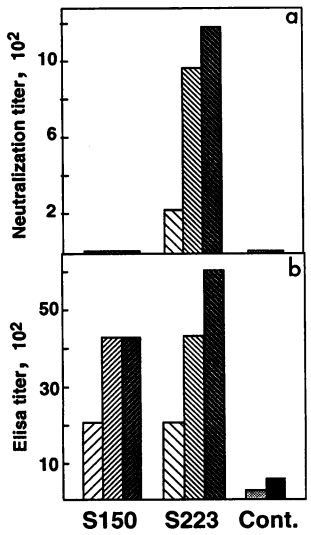

FIG. 4. Virus-binding and -neutralizing antibodies induced by baculovirus-expressed S223 and S150 proteins. The sera from three mice immunized with S223 or S150 antigen were tested in a TGEV neutralization assay using limiting dilutions (a) or in an ELISA using purified virions (b). Two control (Cont.) samples consisted of a preimmune serum (shaded bar) and a serum from a mouse immunized with baculovirus-expressed nonstructural protein TGEV open reading frame 3a, (dark bar), using the same procedure (see Materials and Methods) as for the S-protein derivatives.

was detected, whereas capture of the S150 species was less efficient overall than capture of S223 (not shown).

Antibody response to S223 and S150 proteins. BALB/c mice were immunized with Sf9 cells infected with AcS223 or AcS150 virus to determine whether they would induce antibodies recognizing the authentic S protein. Secreted S150 and S223 proteins were both immunoprecipitated by each of the resulting sera (not shown). The sera raised against S223 neutralized the TGEV infectivity efficiently, with titers ranging from 250 to 1,200 (Fig. 4a), comparable to those of antibodies raised against baculovirus-expressed S protein (21). In contrast, none of the sera from S150-immunized mice exhibited a detectable neutralizing activity (<10), although they reacted with virion-associated S protein at a level equivalent to that of the anti-S223 sera in an ELISA (Fig. 4b). These data indicated that truncation of the C-terminal domain of S223 by about 70 amino acid residues essentially abolished the capacity to induce neutralizing but not binding antibodies.

Binding activity of TGEV S protein for baculovirus-expressed APN. As prevention of attachment to cells is a major mechanism for virus neutralization, it was of interest to determine whether the domain of TGEV S protein bearing most of the neutralization epitopes was involved in binding to the APN receptor. To this end, porcine APN was expressed by using a recombinant baculovirus, and an assay was designed to examine possible interactions between solubilized, recombinant S and APN proteins. Figure 5 shows the result of a representative experiment in which extracts prepared from Sf9 cell cultures labeled following single or dual infection with the relevant baculoviruses were subjected to immunoprecipitation using anti-S polyclonal antibodies. Baculovirus-expressed APN was recognized by several anti-APN MAbs, including G43, and appeared essentially as a single uncleaved band, like APN expressed in mammalian cells (8). The lower apparent molecular mass of APN produced in lepidopteran cells, 120K instead

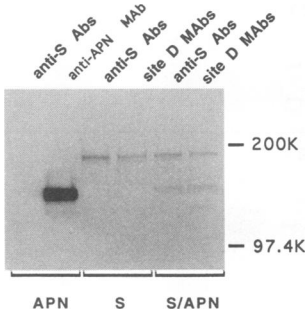

FIG. 5. Interaction between TGEV S and APN recombinant proteins. ^{35}S-labeled cell lysates from cultures infected by AcAPN or AcS recombinant baculovirus or coinfected by these viruses (S/APN) were subjected to immunoprecipitation with a polyclonal anti-S antibody, an anti-APN MAb (G43), or site D MAbs (40.1 and 78.17). Immunoprecipitated polypeptides were visualized by SDS-PAGE (8% gel) and autoradiography.

of 150K, is likely to correlate with the absence of complex glycosylation. As can be seen, a band with a similar molecular mass coimmunoprecipitated with S material when extracts from coinfected, but not singly infected, cultures were incubated with either polyclonal or anti-site D antibodies. The molar ratio between S and APN monomers was estimated to range from 0.5 to 1 in this experiment in which APN was in excess. No coimmunoprecipitation was observed when anti-APN MAbs were used instead of anti-S antibodies (not shown in Fig. 5). This was expected, since the anti-APN MAbs had been selected on the basis of their receptor-blocking activity (6). When extracts from cultures dually infected with baculoviruses expressing APN or sM (small transmembrane protein encoded by TGEV [20]) were used as an additional control, no APN was coimmunoprecipitated by an anti-sM MAb (not shown). From these data, it was concluded that recombinant APN was able to bind TGEV S protein specifically.

Interaction between APN and truncated S proteins. Next, the same kind of assay was performed with one each of the four truncated S derivatives instead of full-length S protein. These experiments clearly showed that APN coimmunoprecipitated with either the S223 or S150 protein (Fig. 6). The S223 protein bound approximately one-third of the material immunoprecipitated by the most potent anti-APN antibody, G43. The S150 species bound consistently less APN material. No conclusion could be drawn concerning a possible interaction

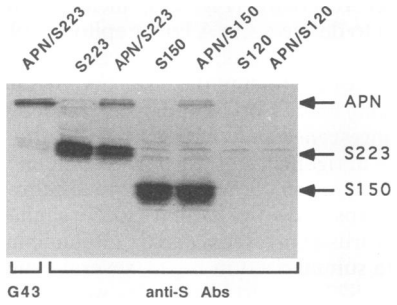

FIG. 6. Interaction between TGEV S-derived and APN baculovirus-expressed proteins. Extracts from labeled cell cultures singly or dually infected as indicated were subjected to immunoprecipitation with either anti-APN MAb G43 or an anti-S polyclonal antibody. Immunoprecipitated material was visualized as in Fig. 2.

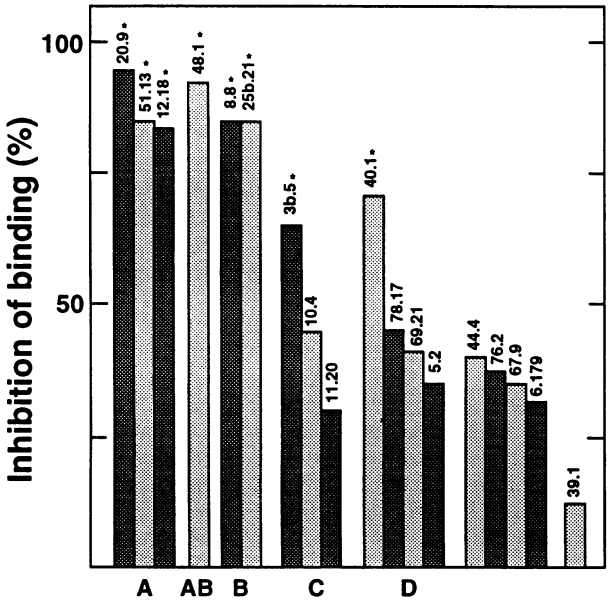

FIG. 7. Inhibition of virus attachment by anti-S MAbs. Purified labeled virions were incubated with the indicated MAbs and then added to isolated ST cell membranes spotted onto a nitrocellulose sheet as described in Materials and Methods. The results are expressed as percent inhibition of binding by reference to virus bound in the absence of MAb (300 to 400 cpm). Strongly neutralizing MAbs (25) are marked with asterisks.

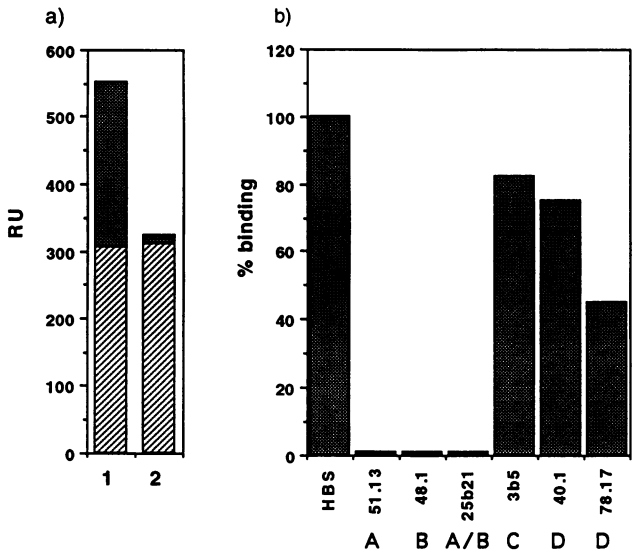

FIG. 8. Blocking of S-APN interaction by neutralizing MAbs. (a) Binding of APN to S1 protein as analyzed by BIAcore. Baculovirus-expressed S1 protein was immobilized onto the biosensor surface by using MAb 44.4, and then cell extracts (see Materials and Methods) from cultures infected with AcAPN (column 1) or a nonrecombinant (column 2) baculovirus were injected. Material bound at the first (hatched blocks) or second (dark blocks) step was quantified in RU. (b) Effects of anti-S MAbs on S1-APN interaction. S1 protein trapped as described above was allowed to react with the indicated antibodies or control buffer (HBS) prior to injection of the APN-containing preparation. Results are expressed as percent binding by reference to APN bound after HBS injection.

between APN and S122 or S88 since, as mentioned above, none of these species were immunoprecipitated by the polyclonal or monoclonal antibodies used. An anti-APN polyclonal antibody was used as an alternative to anti-S antibodies. Only weak immunoprecipitation of the S223-APN complex was observed, suggesting that a large proportion of immunodominant APN epitopes are masked within the complex (not shown). Taken together, these data established that a recombinant polypeptide derived from the CO-26K domain, representing 10 to 15% of the whole S polypeptide chain, retained a substantial capacity to bind APN.

Inhibition of S-APN complex formation by neutralizing antibodies. The anti-S MAbs used in this study differed widely in their effects on virus attachment. Only the MAbs belonging to neutralization sites A and B were able to inhibit the binding of virus to membranes from susceptible cells by more than 85% (Fig. 7). Binding inhibition by MAbs having little or no neutralizing activity did not exceed 45%. Two neutralizing MAbs (3b.5 and 40.1) directed to epitopes outside sites A and B showed intermediate blocking activity. It was thus of interest to examine the effects of neutralizing MAbs on the formation of the S-APN complex. Experiments were performed in the BIAcore format by using baculovirus-expressed S1 and APN proteins. By capturing S1 with a non-AB MAb and then injecting an APN-containing extract, a significant interaction could be evidenced, with a binding stoichiometry of 0.8 mol of dimeric APN per mol of S1 under saturating conditions (Fig. 8a). Specificity was further demonstrated when different anti-AB MAbs were injected and associated to S1 prior to injection of APN (Fig. 8b). Strikingly, anti-A or B MAbs abolished APN binding, whereas anti-C or D MAbs inhibited binding only partially. These results, which confirmed our earlier observation that site A MAbs blocked the binding of intestine APN to coated, purified virions (unpublished data), indicated that antibody neutralizing activity is strongly correlated with inhibition of the formation of the S-APN complex.

Binding of neutralizing antibodies to the S223-APN complex. A last series of experiments was performed to determine whether, as could be expected from the observations presented above, the epitopes recognized by neutralizing antibodies were masked within the S-APN complex. Figure 9a shows the result of an assay in which S223-APN complexes obtained from dually baculovirus-infected cultures were immunoprecipitated with neutralizing MAbs instead of anti-S polyclonal antibody. Both MAbs 51.13 and 25b.21, each representative of the A and B sites, precipitated S223-APN complexes as efficiently as polyclonal antibodies, whereas no complex was detected with an anti-APN MAb. Identical results were obtained with the other site A or B MAbs, or recombinant S protein instead of S223 (not shown). However, when 51.13 MAb was allowed to react with S223 protein prior incubation with APN protein, no complex was immunoprecipitated, thus confirming the blocking of S-APN association by neutralizing MAbs (Fig. 9b). As a positive control, coincubation of S223 and APN proteins obtained from cultures separately infected by the relevant baculovirus led to the formation of complex which was precipitated by the neutralizing MAb (in that case, the APN and S223 molecules were allowed to interact only after solubilization in lysis buffer, which may explain the repeatedly observed low amount of complex). From these data, it was inferred that the interaction of S with APN still leaves accessible the neutralizing epitopes.

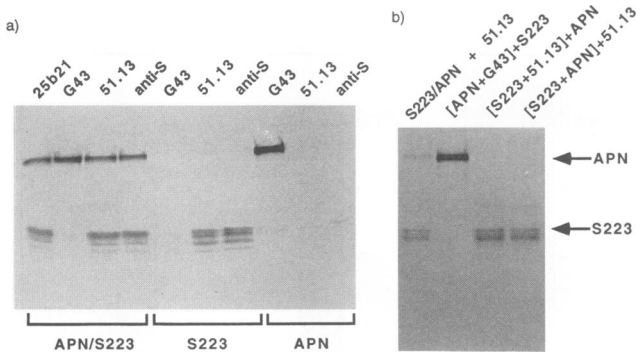

FIG. 9. Recognition and blocking of the S223-APN complex by anti-TGEV neutralizing MAbs. (a) Lysates from labeled cell cultures, singly or dually infected by recombinant baculovirus as indicated, were subjected to immunoprecipitation with anti-APN MAb G43, anti-S MAb 25b.21 or 51.13, or a polyclonal anti-S antibody. Immunoprecipitated material was visualized by discontinuous, bipartite SDS-PAGE (10 to 15% gel) and autoradiography. (b) Immunoprecipitation was performed in two steps, each consisting of a 1-h incubation at 37°C. Brackets identify the first step. Cell extracts from singly infected cultures were used throughout, except for one positive control using an S223-APN dually infected culture (left). The samples were processed as for panel a.

DISCUSSION

This study allowed the delineation of a functional domain of TGEV spike protein which appears to be primarily involved in both virus neutralization and attachment to target cells. In an earlier study, a 26K proteolytic fragment cleaved from native S protein was shown to express a group of epitopes mapped within the two closely related sites A and B, together defining the major neutralization-mediating domain of the virus (9, 12). Such an observation was suggestive of the 26K region being an independent domain. Further evidence of this was provided here through the analysis of the antigenic and immunogenic properties of several derivatives of the relevant region, expressed in the baculovirus system (Fig. 1). Two recombinant proteins, S223 and S150, representing 223- and 150-amino acid-long, amino-coterminal stretches starting at serine 506 of the mature S polypeptide, were found to express the whole set of site A and B epitopes, in contrast to shorter S derivatives (S122 and S80). The immunoreactivity pattern of the S223 and S150 proteins appeared to be essentially similar to that of baculovirus-expressed S1 or S protein, as assayed by precipitation and fluorescence (Fig. 2 and 3). Moreover, the topological relationship between the A and B epitopes, as deduced from an MAb-binding interference map performed with S223 protein (Table 1), was in agreement with that previously found with virion-associated S protein. Finally, both S223 and S150 proteins induced virus-binding antibodies (Fig. 4).

Together, these findings supported the conclusion that the conformations of the S223 and S150 proteins do not profoundly differ from that of the S protein in its native state. Two points need further comments. First, there is a discrepancy between these results and our earlier finding that the baculovirus-expressed protein St, which lacked the transmembrane and cytoplasmic domains only, carried altered A and B antigenic sites (21). This observation likely reflected a cloning artifact, since a S1 construct made from St DNA encoded a product with similarly altered antigenicity (data not shown), unlike the S-derived S1 construct. Second, the S223 and S150 species essentially were not recognized by any of the non-A, non-B anti-S MAbs, thus confirming our earlier topographical mapping. A major exception, however, was MAb 78.17 (site D), which exhibited some reactivity toward both species. This was not unexpected, since PRCV, which encodes a truncated S protein lacking the site D (33), was shown to retain some reactivity toward this but not the other site D MAbs (26). These results are best explained by MAb 78.17 recognizing an epitope assembled from residues located both in the N-terminal and S150 regions of the S polypeptide chain. This would imply that the TGEV spike protein conformation involves interactions between regions widely separated on the primary structure.

Another significant achievement of this work is the demonstration that the S223 recombinant protein binds specifically and efficiently the APN molecule, which acts as a major receptor for TGEV entry into cells (8). Indeed, coinfection of insect cells with recombinant baculoviruses expressing both of these molecules consistently led to the formation of a complex which could be detected by immunoprecipitation with anti-S antibodies and PAGE analysis (Fig. 6). These data provide the first direct evidence for the TGEV spike protein being a receptor-binding protein. Moreover, they establish that determinants sufficient for receptor binding reside within the S amino acid stretch from Ser-506 to Ile-728. This location is consistent with our earlier observation that PRCV binds APN too, thus making unlikely an involvement of the TGEV S N-terminal region into this function (9). Also, it is worth noting that the S223 region is homologous with a region of mouse hepatitis virus which is unlikely to contain a receptor-binding site, since it is polymorphic and shows up to 160-residue-long deletions (30). The observed efficiency of binding suggests that the TGEV S223 region would be primarily involved in S-APN interaction. However, a precise comparison of the respective avidities of the S223 and full-length S proteins for APN will be needed to formally exclude the possibility that additional residues may contribute to this interaction and to confirm that oligomerization of the S polypeptide is not a requirement for APN binding, as was indicated by the data. The S150 species also bound APN, although to a lesser extent than S223. Whether this reflects an inaccurate folding or the loss of a few contact residues remains undetermined.

The data presented here provide further evidence that anti-TGEV neutralizing antibodies may prevent attachment to host cells. Among a panel of 18 IgG MAbs, only those with strong neutralizing activity were able to inhibit efficiently the binding of radiolabeled particles to isolated cell membranes (Fig. 7), in agreement with another study in which an independent set of MAbs and intact cells was used (38). This finding prompted us to investigate the effects of neutralizing antibodies with respect to the interaction between TGEV S protein and the APN receptor. The main features were as follows: (i) three MAbs representative of the A and B sites completely blocked the binding of solubilized recombinant APN to TGEV S1 protein immobilized by an appropriate MAb in the BIAcore format (Fig. 8); (ii) all of the neutralizing site A and B MAbs were found to recognize a preformed S223-APN complex in an immunoprecipitation assay, in striking contrast to anti-APN MAbs, which either did not recognize or displaced this complex (Fig. 9a); (iii) in a similar assay, the S223 protein preincubated with neutralizing MAbs no longer recognized APN (Fig. 9b), consistent with the BIAcore data. Taken together, these results strongly support the idea that prevention or impairment of the cellular receptor recognition is one major mechanism involved in the neutralization of TGEV, as already postulated for some other viruses (13). On this basis, neutralizing antibodies may act in two different ways: (i)

blocking of virus attachment to cells or (ii) blocking of the recruitment of receptor molecules, a step presumed to be necessary for virus endocytosis and/or fusion. The latter notion predicts that fully neutralized virions should still bind to cells at a certain antibody-to-virus ratio, a situation which was actually observed in a detailed study of TGEV neutralization (38).

Notwithstanding their blocking activities, the site A and B antibodies recognized S223 protein when complexed with APN. The existence of multivalent complexes of the two coexpressed proteins could explain this observation. However, this possibility seems to be unlikely because (i) the anti-APN MAbs did not bind the complexes and (ii) the S223 protein was essentially in a monomeric form, according to the BIAcore data. Therefore, this finding strongly suggests that the S determinants involved in the binding of receptor and of neutralizing antibodies, respectively, are essentially distinct. This would be consistent with the fact that neutralization-resistant mutants previously selected in our and another laboratory generally grew as efficiently as the parental virus (7, 22). The amino acid substitutions identified so far in the S polypeptide encoded by these mutants are all clustered within the amino-terminal third of the S223 sequence (Fig. 1). Hence, it can be speculated that the receptor-binding site is located downstream of the region where the site A and B epitopes are putatively located and within the stretch from Asp-579 to Ser-655 common to S223 and S150. This region, which is highly conserved among TGEV, feline infectious peritonitis virus, and canine coronavirus, may contain residues contributing to receptor binding, with a few residues possibly imparting the species specificity of the recognition. Inspection of the sequence has revealed no particular feature except the absence of potential glycosylation sites and a strongly predicted amphiphilic β sheet around residues 615 to 635, which is lost in the S122 derivative.

The apparent ability of the neutralizing antibodies to recognize the S-APN complex also raises the question of the nature of the interactions which lead to masking of the receptor-binding determinants in the antibody-bound S protein. The observation that the S150 protein induced antibodies with virus-binding but no neutralizing activity (Fig. 4) could be relevant in this regard, as it points to the possibility that a structural element of the S223 C-terminal region is required for masking. This element might consist of one or a few additional residues bound by an antibody so that it bridges over the receptor-binding site. Alternatively, the masking of the receptor-binding determinants might result from an induced alteration of the S-protein conformation. The possible occurrence of transconformational events following interaction with site A or B antibodies has already been proposed on the basis of competition binding analyses (7). Antibodies to an inaccurately folded S150 form would be unable to trigger the postulated conformational changes. These findings overall support the view that the S223 region has a relatively complex structure. The availability of this domain as a soluble, self-functioning molecule, which could be more easily amenable to crystallization than the whole S protein, should allow a precise analysis of the interaction between the virus attachment protein and the cellular receptor through the determination of the three-dimensional structure of the S223-APN complex.

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