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Single Amino Acid Changes in the Viral Glycoprotein M Affect Induction of Alpha Interferon by the Coronavirus Transmissible Gastroenteritis Virus

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Transmissible gastroenteritis virus, an enteropathogenic coronavirus of swine, is a potent inducer of alpha interferon (IFN-α) both in vitro and in vivo. Previous studies have shown that virus-infected fixed cells or viral suspensions were able to induce an early and strong IFN-α synthesis by naive lymphocytes. Two monoclonal antibodies directed against the viral membrane glycoprotein M (29,000; formerly E1) were found to markedly inhibit virus-induced IFN production, thus assigning to M protein a potential effector role in this phenomenon (B. Charley and H. Laude, J. Virol. 62:8–11, 1988). The present report describes the selection and characterization of a collection of 125 mutant viruses which escaped complement-mediated neutralization by two IFN induction-blocking anti-M protein monoclonal antibodies. Two of these mutants, designated H92 and *dm*49-4, were found to exhibit a markedly reduced interferogenic activity. IFN synthesis by lymphocytes incubated with purified suspensions of these mutants was 30- to 300-fold lower than that of the parental virus. The transcription of IFN-α genes following induction by each mutant was decreased proportionally, as evidenced by Northern (RNA) blot analysis. The sequence of the M gene of 20 complement-mediated neutralization-resistant mutants, including the 2 defective mutants, was determined by direct sequencing of genome RNA. Thirteen distinct amino acid changes were predicted, all located at positions 6 to 22 from the N terminus of the mature M protein and within the putative ectodomain of the molecule. Two substitutions, Thr-17 to Ile and Ser-19 to Pro, were assumed to generate the defective phenotypes of mutants *dm*49-4 and H92, respectively. The alteration of an Asn-Ser-Thr sequence in *dm*49-4 virus led to the synthesis of an M protein devoid of a glycan side chain, which suggests a possible involvement of this structure in IFN induction. Overall, these data supported the view that an interferogenic determinant resides in the N-terminal, exposed part of the molecule and provided further evidence for the direct role of M protein in the induction of IFN-α by transmissible gastroenteritis virus. The acronym VIP (viral interferogenic protein) is proposed as a designation for this particular class of proteins.

Alpha interferon (IFN-α) can be induced after contact of leukocytes with viruses, bacterial products, or tumor cells (8). In contrast to IFN-β, which is produced after viral infection of various cell populations and for which a critical induction factor is RNA, IFN-α is induced in hematopoietic cells by a distinct mechanism (11, 19). Indeed, inactivated viral particles as well as glutaraldehyde-fixed virus-infected cells can induce IFN-α (1, 2, 4, 10, 11, 13, 19). These findings indicate that virus replication is not required for IFN-α induction. Instead, they suggest that membrane interactions between interferon producer cells (IPC) and viral proteins present at the viral particle surface or at the virus-infected cell membrane provide stimuli to induce expression of IFN-α genes.

The nature of the IPC population is not completely elucidated. Recent reports indicate that, in response to herpes simplex virus, human IPC are an infrequent but highly efficient mononuclear leukocyte population (3, 10, 26). These cells are characterized as nonadherent, non-T, non-B cells (18, 24) expressing major histocompatibility complex class II (9, 22, 23, 29) and CD4 (30) molecules at their membrane. Several identical phenotypic features are shared by murine and human IPC in response to other stimuli such as influenza virus (25), Dengue virus (12), or mycoplasmas (2). For transmissible gastroenteritis virus (TGEV), a coronavirus which induces acute diarrhea and intense IFN-α production in newborn piglets (14), we have also shown that IFN-α is rapidly produced following exposure of non-adherent, non-T, non-B, major histocompatibility class II^+, $CD4^+$ porcine blood leukocytes to virus-infected cells (5).

Strikingly, very few studies have been aimed at understanding the nature of the viral component interacting with leukocytes to induce IFN-α in vitro. IFN-α induction by herpes simplex virus was reported to be inhibited by monoclonal antibodies (MAbs) against the major viral glycoprotein D (17), which argues for a critical role of this molecule. In similar experiments, we examined the effects of MAbs directed at the three different TGEV structural proteins on IFN-α induction after exposure of leukocytes to virus-infected cells. It appeared that IFN-α induction was only blocked by two MAbs directed at the transmembrane glycoprotein M (formerly named E1), whereas MAbs specific for the nucleocapsid protein or for the spike glycoprotein S had no such effects (4). These data strongly suggested that IFN-α induction could result from interactions between leukocytes and a defined domain of one viral glycoprotein, namely, M protein.

The objective of the present study was to determine the putative IFN-inducing domain of the TGEV M glycoprotein. This was done by selecting epitope mutant viruses that were no longer recognized by either of the two MAbs previously

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TABLE 1. Properties of the MAbs to the M protein of TGEV used in this study

MAb (isotype)	Blocking of IFN induction[a]	CMN[b]	Membrane immunofluorescence[c]	Competition for binding[c] with:
49 (ND[d])	+	+	+	25 and 9
25 (G2a)	+	+	+	9
9 (G1)	−	−	+	25
3 (G3)	−	−	−	None

[a] Data from Charley and Laude (4).
[b] This study.
[c] From Delmas and Laude (7b).
[d] ND, not determined.

shown to block IFN induction. Viruses thus selected were then individually examined for their interferogenic activity. Sequencing the M protein gene of several of these mutants allowed us to identify amino acid changes that markedly altered the ability of the virus to induce expression of IFN-α genes.

MATERIALS AND METHODS

Virus production and purification. The high-passage Purdue-115 strain of TGEV was used as a virus source. The procedures for virus propagation in the pig kidney cell line PD5 and titration of infectivity in the swine testis ST cell line have been reported previously (15). Virions were purified by one-step rate zonal centrifugation essentially as described previously (15); the quantities of virus in suspensions were calculated from UV absorbance values.

MAbs. The murine MAbs directed against TGEV M protein (formerly E1) have been characterized previously (15), and their respective properties are described in Table 1. The complete designation of the MAbs was 25.22, 49.22, 9.34, and 3.60 in previous studies. Heat-inactivated ascites fluids were used as a source of antibodies.

Selection of CMN-resistant mutants. Resistance to complement-mediated neutralization (CMN) was used as a mean to select M protein mutant viruses. The epitope mutants were obtained through three or four cycles, each consisting of neutralization of virus suspensions and then multiplication of the surviving fraction. Most of the experiments were done in 96-well microplates maintained in a CO_2 incubator. Wells containing 10^7 to 10^8 PFU of virus (100 μl of virus suspension) combined with the appropriate MAbs (100 μl of minimal essential medium plus ascites fluid diluted 1:25) were incubated for 1 h at 38°C. After addition of newborn rabbit serum (NRS) at a dilution of 1:50 as a source of complement, wells were incubated for one additional hour at 38°C; then the suspensions were transferred onto ST cell monolayers established in microplates. One hour later, the surrounding medium was replaced by minimal essential medium plus 5% newborn calf serum, and the plates were allowed to stay at 38°C. The cultures were frozen as cytopathic effect became evident. After several cycles following the same procedure, virus clones were isolated by plaque purification and assayed for infectivity, CMN resistance, reactivity in indirect immunofluorescence tests, and IFN induction.

CMN assay. Resistance of the mutants to CMN was measured in a plaque reduction assay performed in ST cells as for a standard TGEV neutralization assay (15), except that virus-MAb mixtures were incubated for an additional 60 min at 38°C in the presence of NRS (1:100). The CMN index was defined as $\log_{10}$ titer (virus + MAb) − [$\log_{10}$ titer (virus + MAb + NRS) − $\log_{10}$ titer (virus + NRS)]. Inactivation of virus infectivity by NRS in the absence of MAb (nonspecific CMN) was ≈0.3 $\log_{10}$.

Immunofluorescence assay. Indirect immunofluorescence was performed on acetone-alcohol-fixed cells as described previously (15).

PBMC. Porcine peripheral blood mononuclear cells (PBMC) were obtained from heparinized blood by Ficoll density centrifugation on MSL (density, 1.077; Eurobio, Paris, France). PBMC were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics.

IFN induction. PBMC were induced to produce IFN-α by overnight incubation on TGEV-infected glutaraldehyde-fixed cell monolayers as described previously (4). Briefly, PD5 cells were plated in 96-well microplates, infected with TGEV for 18 h at a multiplicity of infection of 1, and then fixed with 0.25% glutaraldehyde (1 h at 4°C) and stored with 3% glycine. Monolayers were washed before the addition of PBMC (100 μl per well containing 5×10^5 cells). Supernatants were collected after 18 h of incubation at 37°C and assayed for IFN activity. Alternatively, PBMC were induced by overnight incubation with suspensions of purified virus.

IFN bioassay. Serial $\log_3$ dilutions of supernatants from induced PBMC were assayed for IFN on bovine MDBK cells using vesicular stomatitis virus as a challenge (14). A standard porcine IFN-α was included in each assay. This standard was calibrated on MDBK cells with the human international reference IFN B69/19 (National Institutes of Health, Bethesda, Md.). In our results, 1 U is equivalent to 1 IU of human IFN.

Northern (RNA) blot analysis. Total RNA was isolated by the guanidium thiocyanate method (7) from PBMC following incubation with 300 ng of purified virions per 10^8 cells for 6 h. Twenty micrograms of each sample was examined by Northern blot analysis. Electrophoresis was done on a 1% agarose gel which was blotted onto nylon (Hybond N^+; Amersham). The IFN-α probe used was a 0.85-kb *Eco*RI-*Hpa*I genomic fragment containing the complete coding sequence of porcine IFN-α1 gene provided by F. Lefèvre (20). Hybridization was performed at 65°C in hybridization buffer (1 mM EDTA, 7% sodium dodecyl sulfate [SDS], 5 g of nonfat milk per liter), and blots were washed at 55°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS. A positive control for hybridization consisted of an actin probe provided by G. Alm.

Direct RNA sequencing. Genomic RNA was prepared from virions semipurified by ultracentrifugation through a 25% glycerol cushion. RNA was extracted with phenol-chloroform after treatment of virions with proteinase K plus SDS. After precipitation with 2 M LiCl, RNA was resuspended in water and heat denatured (100°C for 1 min). Reverse transcription was performed as originally described by Zimmern and Kaesberg (31). Priming was done with 25-mer synthetic oligonucleotides complementary to the TGEV M sequence (16). The following oligonucleotides (synthesized on a Biosearch 8600 apparatus) were used: 5′ CTTCCATATTGTAG CACAGTTATAA; 5′ ACATAATCCAGAGTACAAATG TAAC; 5′ TTGAACCCTTCAGCGTACAAATTCC; 5′ TTG TCCCTGGTTGGCCATTTAGAAG. The elongation reaction was performed at 42°C for 20 min with 1 to 2 μg of RNA per sequence, avian myeloblastosis virus reverse transcriptase (Appligène), ^{35}S-dATP (Amersham), and a deoxynucleotide/dideoxynucleotide ratio of 3.75:1, 5:1, 6:1, and

12.5:1 for nucleotides T, C, G, and A, respectively. The samples were analyzed on 6% acrylamide–8 M urea gels.

Analysis of labeled viral polypeptides. Preparation of [^{35}S]methionine-labeled cytosols, immunoprecipitation assays, and SDS-polyacrylamide gel electrophoresis (PAGE) analysis were performed as reported previously (15).

RESULTS

Isolation of M protein antigenic mutant viruses. Epitope mutants were selected to localize the region of the M molecule recognized by the two MAbs (25 and 49) that are able to block IFN induction. Moreover, it was reasoned that at least part of the mutations leading to the loss of the corresponding epitopes on M protein might affect its IFN-inducing capacity. While anti-TGEV M protein MAbs have essentially no direct neutralizing activity (15), it turned out that both MAbs 25 and 49 efficiently neutralized virus infectivity in the presence of complement. The CMN index of the wild-type Purdue virus reached 3 ± 0.2 ($\log_{10}$) with either of the two MAbs under the conditions of our assay. This provided the positive pressure for selection of mutants.

A collection of independent mutants was established through three or four rounds of enrichment of the virus population surviving CMN and then plaque purification. Virus clones considered as being MAb resistant had a CMN index ranging from 0.1 to 1 toward the selector MAb(s) and/or exhibited no antigenic reactivity in indirect immunofluorescence tests at an MAb dilution of 1:20. The 125 mutants selected were classified into three groups depending on the selection procedure used: (i) 12 and 9 mutants selected against either MAb 25 or 49, respectively; (ii) 96 mutants selected against the two MAbs used concomitantly; (iii) 8 mutants selected against the two MAbs used sequentially.

IFN induction by epitope mutants. The IFN-inducing capacity of each mutant was tested by exposure of PBMC to glutaraldehyde-fixed, virus-infected cells or by direct contact with viral suspension. A majority of the single mutants (groups i and ii) induced amounts of IFN equivalent to those of the parental virus; this interferogenic activity was no longer inhibitable by MAbs 25 and/or 49. However, the mutant designated H92 induced substantially lower IFN titers than the parental virus or other mutants. As shown in Table 2, H92 virus-infected cells induced from 88 to 99% less IFN-α than Purdue-115 virus-infected cells. Similarly, IFN-α induction by clarified viral suspensions of H92 virus applied to PBMC (1 PFU per cell for 16 h at 37°C) was 1 to 4% of that of wild-type virus (three experiments, data not shown). A second mutant with decreased IFN-inducing capacity (*dm*49-4) was identified among the set of double mutants (group iii). This mutant consistently induced about 1% of the IFN induced by the other mutants and the wild-type strain virus (Table 2). It was necessary to confirm that the observed defect was not due simply to a decreased synthesis of M protein within H92 or *dm*49-4 virus-infected cells. Therefore, IFN induction was measured after exposure of PBMC to equivalent amounts of sucrose gradient-purified preparations of each mutant or of wild-type virus. As a result, the interferogenic activity of H92 and *dm*49-4 viruses was found to be 30- to 300-fold lower than that of the wild-type virus (Fig. 1). This strongly suggested that the altered interferogenic capacity of both mutants reflected qualitative rather than quantitative modifications of M protein.

In addition, it was shown by Northern blot analysis that total RNA preparations from H92- or *dm*49-4-induced PBMC contained markedly less IFN-α mRNA than wild-type virus-induced PBMC extracts, whereas the different PBMC extracts tested contained equal amounts of actin mRNA (Fig. 2). Moreover, IFN-α mRNA synthesis appeared to be lower with *dm*49-4 than with H92, which is consistent with the relative IFN titers measured in the same experiment. It was concluded from these data that both mutants induced transcription of the IFN-α gene(s) less efficiently than the parental virus.

TABLE 2. IFN-α-inducing properties of TGEV mutants H92 and *dm*49-4 compared with the Purdue-115 wild-type strain

Expt no.	IFN induction[a]		%[b]
	Mutant	Wild type	
	H92		
1	30	3,000	1
2	40	3,000	2
3	4	110	4
4	30	3,000	1
	*dm*49-4		
1	7	5,200	0.1
2	110	15,000	0.5
3	110	9,000	1

[a] PBMC (5×10^6/ml) were incubated overnight at 37°C on virus-infected, glutaraldehyde-fixed PD5 cell monolayers (as described in Materials and Methods). Each experiment was performed with PBMC isolated from a different animal. IFN activity (units per milliliter) of PBMC supernatants is shown.

[b] (Mutant-induced IFN titer/wild type-induced IFN titer) × 100.

Sequence analysis of M gene epitope mutants. The sequence of the 200 5′-most nucleotides of the M gene from 20 CMN-resistant mutants, including H92 and *dm*49-4 mutants, was determined by direct sequencing of genomic RNA and compared with the sequence of the parental Purdue-115 virus (16). The established sequences covered the signal peptide, the N-terminal hydrophilic stretch of about 30 amino acids presumed to protrude from the outer lipid membrane, and part of the first transmembrane segment. All the mutants selected against a single MAb or the two MAbs

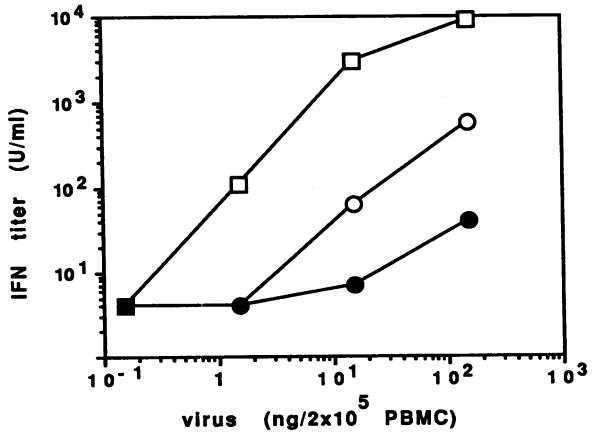

FIG. 1. Induction of IFN-α by virion preparations of wild-type and mutant TGEVs. PBMC (2×10^5 in 200 μl) were incubated overnight at 37°C with different amounts of sucrose gradient-purified virus (1 ng ≃ 3×10^6 particles). IFN activity was assayed from PBMC supernatants. Symbols: □, Purdue-115; ○, H92; ●, *dm*49-4.

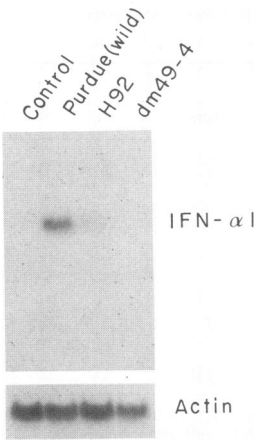

FIG. 2. Induction of IFN-α mRNA in lymphocytes by wild-type (Purdue) or mutant (H92 and *dm*49-4) viruses. Total RNA extracted from PBMC virus induced for 6 h (300 ng of purified particles per 10^8 PBMC) was analyzed by Northern blotting with a ^{32}P-labeled probe specific for IFN-α1. A positive control hybridization with an actin probe is shown. IFN titers in virus-induced PBMC supernatants were 1,000, 20, and 4 IU/ml for wild-type (Purdue), H92, and *dm*49-4 viruses, respectively. Control consisted of mock-induced PBMC.

FIG. 3. Location of the amino acid changes in the M protein of TGEV mutants. The sequence of the N terminus of TGEV M protein is shown (16) (cleaved signal peptide sequence omitted). The N-linked glycosylation site is boxed. Charged residues are circled. Cysteines residues are underlined. The predicted first transmembrane segment is underlined (bold). The positions of the deduced amino acid changes (Table 3) are marked by symbols above the sequence, using open symbols for those found in IFN induction-defective mutants H92 (□) and *dm*49-4 (○).

concomitantly showed a single nucleotide substitution. An additional nucleotide substitution was found in all double mutants resulting from a sequential selection with the two MAbs. The location and nature of the predicted amino acid changes are displayed in Fig. 3 and Table 3. The changes were distributed among eight different positions, all between amino acids 6 and 22 from the N terminus of the mature protein. A change from Ser to Pro at position 19 was predicted for the M protein encoded by mutant H92. The M protein of mutant *dm*49-4 was predicted to contain an amino acid-conservative change of Glu to Asp at position 22 (as was also found for mutant 25.1 from which *dm*49-4 was derived) and a second change of Thr to Ile at position 17, within the potential glycosylation site Asn-Ser-Thr. Complete sequencing (786 nucleotides) of the M gene of these two mutants revealed no other difference from the wild-type virus. With the exception of a codon GAT (Asp) instead of GGT (Gly) at position 178, the RNA sequences were identical to the cDNA sequence reported previously (16).

Polypeptide pattern of H92 and *dm*49-4 mutants. PAGE analysis of *dm*49-4 mutant polypeptides revealed that the relative mobility (M_r) of the M protein major species was shifted from 29,000 (Fig. 4, lane 2) to 26,000 (lane 4), which corresponds to the M_r of the deglycosylated or unglycosylated forms of the M protein (7a). This result was consistent with the sequence data predicting the loss of the uniquely accessible Asn-linked glycosylation site. Strikingly, the H92 mutant also exhibited an altered pattern of glycosylation; apparently, a substantial proportion of the M protein molecules (estimated to be 70%) did not undergo glycosylation, leading to the simultaneous presence of both the 26,000- and 29,000-M_r species (Fig. 4, lane 3). These results confirmed that both IFN-defective mutants encoded an M protein with distinctive molecular features.

DISCUSSION

An increasing number of reports describe induction of IFN-α following in vitro exposure of leukocytes to nonin-

TABLE 3. Predicted amino acid changes and interferogenic activity of TGEV M protein epitope mutants

Mutant designation[a]	Epitope[b]				Nucleotide substitution	Amino acid change	Position[c]	Interferogenic activity (%)[d]
	49	25	9	3				
49-12, 49-19	−	+	−	+	AAA–GAA	Lys-Glu	6	100
49-9, 49-10	−	+	+	+	GAT–GAG	Asp-Glu	10	30–100
*dm*49-6 (25-7)	−	−	−	+	TTG–TTT	Leu-Phe	11 (22)	100
49-3	−	+	+	+	AGT–ATT	Ser-Ile	16	30
*dm*25-1 (49-9), *dm*25-8 (49-9)	−	−	−	+	AGT–ATT	Ser-Ile	16 (10)	100
*dm*25-3 (49-12)	−	−	−	+	AGT–AGA	Ser-Arg	16 (6)	100
***dm*49-4 (25-1)**	−	−	−	+	ACA–ATA	Thr-Ile	17 (22)	<1
H92	−	−	−	+	TCT–CCT	Ser-Pro	19	1
*dm*25-9 (49-12)	−	−	−	+	TCT–TTT	Ser-Phe	19 (6)	100
B41	−	−	−	+	GAT–CAT	Asp-His	20	30
D4, E1, 49-4	−	−	−	+	GAT–GCT	Asp-Ala	20	30–100
25-8	−	−	−	+	GAT–TAT	Asp-Tyr	20	100
25-12	+	−	−	+	GAT–AAT	Asp-Asn	20	100
25-1, 25-7	+	−	−	+	GAG–GAT	Glu-Asp	22	30–100

[a] Designation of single mutants selected against one MAb uses the name of the selector MAb (e.g., **49**-11 or **25**-8). Designation of single mutants selected against two MAbs concomitantly uses a capital letter as a prefix (e.g., H92). Designation of double sequential mutants uses the prefix *dm* followed by the name of the second selector MAb; the name of the parental single mutant is given in parentheses.

[b] Expression of epitopes was determined by indirect immunofluorescence with ascites fluids from the four indicated MAbs. +, positive immunofluorescence at a dilution of >1:2,500; −, negative immunofluorescence at a dilution of <1:20.

[c] For *dm* (sequential) mutants, the position of the amino acid change resulting from the first selection is given in parentheses.

[d] (Mutant-induced IFN titer/wild type-induced IFN titer) × 100.

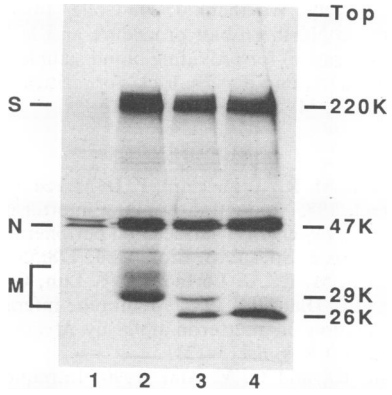

FIG. 4. Polypeptide pattern of TGEV mutants H92 and *dm*49-4. Cell extracts from infected cultures labeled with [^{35}S]methionine were immunoprecipitated by using polyclonal antibodies enriched with MAb 3. Resulting material was resolved by 10% SDS-PAGE and autoradiographed. M_r values of polypeptide species (left) are given (right) (K, $\times 10^3$). Lane 1, mock-infected cells; lanes 2 to 4, cells infected with Purdue wild-type, H92, and *dm*49-4 viruses, respectively.

fectious viral particles or to virus-infected cells fixed with glutaraldehyde. This strongly suggests that IFN-α production results from membrane interactions between the leukocyte cell membrane and an outer exposed inducer structure (8, 11, 19). Attempts to characterize such a structure have consisted of blockage experiments in which MAbs directed at viral antigens were added during the induction phase. Thus, IFN-α induction by HSV could be blocked by an MAb to the viral glycoprotein D (17). Similarly, we have shown that two MAbs directed at the coronavirus TGEV glycoprotein M could block IFN-α induction, whereas MAbs to the nucleocapsid and the spike proteins had no effect (4). The aim of the present study was to provide direct evidence for the critical role played by viral glycoproteins in the induction of IFN-α. The strategy that we used was to generate variant viruses by selection against the anti-M proteins MAbs 25 and 49, previously shown to block IFN induction by TGEV, and then to search for concomitant alteration of the interferogenic properties of these viruses. The results obtained from these experiments allowed us to delineate a domain of the M molecule involved in this biological function.

A panel of 125 single or double antigenic mutants which escaped CMN by either one or by both MAbs 25 and 49 was established. Most (i.e., 123) of these mutants were found to induce as much IFN-α as the wild-type strain (Purdue-115) virus. These data indicate that although binding of MAb to epitopes 25 and 49 could block IFN-α induction, these structures are not, by themselves, the actual IFN-inducing determinants. Such a finding was not unexpected since a third anti-M protein MAb (named 9), also shown to exert a reciprocal competitive binding with MAb 25 (Table 1), did not block induction of IFN (4). However, two mutants, designated H92 and *dm*49-4, exhibited a markedly reduced ability to induce IFN-α (Table 2). The defect in IFN induction was observed when PBMC were exposed to glutaraldehyde-fixed virus-infected cells but also when they were exposed to equivalent amounts of purified viral particles (Fig. 1). The latter result rules out the possibility that low IFN induction by H92 or *dm*49-4 virus-infected cells is simply due to a reduced amount of M protein exposed at the surface of the inducer cells. IFN induction experiments with purified particles established in addition that the TGEV virion is an extremely potent inducer since IFN synthesis was still detected at an estimated ratio of wild-type particles to PBMCs of 1/1. The defect in IFN-α induction by H92 or *dm*49-4 virus was evidenced not only at the protein synthesis level, as measured by antiviral activity in PBMC supernatants, but also at the IFN-α gene transcription level, as determined by Northern blot analysis (Fig. 2).

The above results directly confirmed the role played by TGEV M glycoprotein in IFN-α induction. It was therefore relevant to determine the position and nature of the amino acid changes induced in selected mutants. This was done through direct RNA sequencing of the M gene of 20 of them, including the 2 defective mutants (Table 3). Both selector MAbs were thought to recognize externally exposed epitopes, based on their ability to mediate membrane fluorescence and CMN (Table 1). All the 13 distinct amino acid replacements identified were indeed clustered in a relatively short amino acid stretch at positions 6 to 22 from the N terminus of mature M protein, located within the putative ectodomain of the molecule (Fig. 3). From sequence data (16), the ectodomain of TGEV M protein was predicted to consist of a highly charged sequence of about 30 residues with a unique N-glycosylation site at position 15 (Fig. 3). Our data thus provide further experimental support for the proposed membrane topology of coronavirus M protein. This molecule is assumed to be anchored to the membrane by three consecutive spanning α-helices, leaving only 10% of its N-terminal part exposed at the outer surface; the remaining C-terminal part is located at the inner face of the membrane (28). The present data also give information about the respective topology of the epitopes expressed by the M ectodomain. Examination of the antigenic pattern of the single mutants revealed that mutations conferring an epitope phenotype of 49^- 25^+ or 49^+ 25^- segregated at positions 6, 10, and 16, and 20 and 22, respectively, whereas those leading to a double resistance phenotype (49^- 25^-) involved residues 19 and 20. This distribution supports the view that the relevant epitopes overlap only partially, which is consistent with the nonreciprocal competition of binding observed between the two MAbs (Table 1).

As mentioned above, most mutations altering the MAb binding, even those conferring a double CMN resistance phenotype, caused no change in the interferogenic activity of the virus. The nature of the amino acid replacements which generated IFN-defective properties is thus worth examining. Two amino acid changes were deduced for the *dm*49-4 M protein: Thr to Ile and Glu to Asp at positions 17 and 22, respectively. Since a unique change of Glu to Asp at position 22 did not significantly alter interferogenicity of mutant 25.1 (Table 3), the change at position 17 was presumed to be responsible for the observed defect. The latter change suppresses the Asn-linked glycosylation site, and based on PAGE analysis, *dm*49-4 virus actually encodes an M protein devoid of glycans (Fig. 4). On the other hand, a change of Ser to Pro at position 19 was found for mutant H92. PAGE analysis of the M protein synthesized by this virus revealed that a portion of the polypeptide lacks a carbohydrate chain (Fig. 4). We assume that the incomplete utilization of the acceptor site is due to an unfavorable secondary structure created by the presence at position +4 from Asn of a proline residue, often associated with a bending of the polypeptide chain. A change of Ser to Phe at the same position altered neither the interferogenic activity nor the glycosylation pattern of mutant 25.9 (Table 3 and data not shown).

The finding that both the *dm*49-4 and H92 mutants had a

total or partial defect in M protein glycosylation raises the question of the potential role of the carbohydrate component in the expression of the interferogenic activity. In the context of an interaction between the viral protein and a putative receptor present on IPC, two types of explanations can be put forward. First, addition of the carbohydrate moiety might control proper folding of the polypeptide chain. Second, the oligosaccharide residues might contribute to the stabilization of the complex formed between the effector protein and the IPC during the induction stage. In both situations, an impairment of glycosylation would result in loosened interactions with the cellular receptor. Alternatively, carbohydrates themselves might be the key inducer component. The finding that the *dm*49-4 mutant, which encodes a nonglycosylated M protein, exhibits a residual IFN-inducing activity may argue against this view. Additional studies are needed, however, to clarify this crucially important point. In any case, the present data are consistent with those of our recent study which showed that the glycosylation of the viral proteins, and more specifically the presence of complex-type oligosaccharides, is an important requirement for an efficient induction of IFN-α by TGEV (6). A last point emerging from the present experiments is that the glycosylation defect had no observed consequences on the specific infectivity or multiplication rate of either mutant (data not shown). Such a finding raises the question of the biological significance of the carbohydrate moiety associated with M protein, as this structural feature is conserved among TGEV strains and coronaviruses in general.

An obvious advantage of the approach developed in this study was the isolation of infectious mutant viruses, of which the biological properties can be further explored, both in vitro and in the host animal. An alternate strategy to define the M interferogenic domain would involve site-directed mutagenesis and expression of recombinant M protein in a convenient cell system. Earlier studies on infectious bronchitis virus and mouse hepatitis virus coronaviruses have provided convincing evidence that M protein is targeted to Golgi membranes, where it accumulates in the absence of virus budding (27). Expression of recombinant M protein at the outer cell membrane was only achieved through deletion of the sequences encoding the first two transmembrane domains (21). It is thus probable, even though other mechanisms cannot be excluded, that IFN induction in TGEV-infected cells is essentially mediated by virion-associated M protein. Experiments based on the expression of both intact and truncated TGEV M protein are required to formally answer this question and to establish whether M protein would still be interferogenic in the absence of other virion structural components.

In conclusion, this study further argues for the direct involvement of a viral glycoprotein for the onset of IFN-α gene expression in IFN-producing cells. We believe that sufficient evidence has accumulated now to coin a name for this particular class of proteins; we propose VIP, for viral interferogenic protein. Although some VIPs have already been described, this study is the first to define a functional domain in terms of primary structure. This, together with similar information to be gained on other VIPs, should help elucidate the molecular determinism of this biological phenomenon, which certainly deserves more attention regarding its potential significance for immunity to viral infections.

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