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I. Introduction

Soon after the first isolation of an influenza C virus from a patient (Taylor, 1949), it became obvious that this virus differs from other myxoviruses in several aspects. Pronounced differences have been observed in the interactions between the virus and cell surfaces, suggesting that influenza C virus attaches to receptors different from those recognized by other myxoviruses. While influenza A and B viruses agglutinate erythrocytes from many species, including humans, the spectrum of erythrocytes agglutinated by influenza C virus is much more restricted. Erythrocytes from rats, mice, and adult chickens are suitable for hemagglutination and hemadsorption tests; cells from other species, however, react not at all or only poorly with influenza C virus (Hirst, 1950; Minuse et al., 1954; Chakraverty, 1974; Ohuchi et al., 1978). Differences are also observed so far as hemagglutination inhibitors are concerned. A variety of glycoproteins have been shown to prevent influenza A and B viruses from agglutinating erythrocytes. In the case of influenza C virus, rat serum was for a long time the only known hemagglutination inhibitor (Styk, 1955; O’Callaghan et al., 1980).
A difference in the receptors for influenza C virus and other myxoviruses was also suggested by studies on the receptor-destroying enzyme. The ability of influenza C virus to inactivate its own receptors was reported soon after the first isolation of this virus from a patient (Hirst, 1950). However, the influenza C enzyme did not affect the receptors of other myxoviruses and, conversely, the receptor-destroying enzyme of either of the latter viruses was unable to inactivate the receptors for influenza C virus on erythrocytes. While the enzyme of influenza A and B virus was characterized as a neuraminidase in the 1950s (Klenk et al., 1955), even with refined methodology no such activity was detectable with influenza C virus (Kendal, 1975; Nerome, et al., 1976).

It is now known that both the receptor-binding and receptor-destroying activities as well as the fusion activity of influenza C virus are mediated by the only glycoprotein present on the surface of the virus particle. The structure and functions of this protein, which is designated HEF, are reviewed in the following sections.

II. Structure

A. Primary Structure

For two strains of influenza C virus, the RNA segment containing the genetic information for HEF has been cloned and sequenced (Nakada et al., 1984; Pfeifer and Compans, 1984). Sequence data for several strains have been obtained by direct sequencing of the viral RNA (Buonagurio et al., 1985; Adachi et al., 1989). The gene is 2070–2075 nucleotides in length and can code for a polypeptide of 654–655 amino acids (Fig. 1). The predicted polypeptide has a molecular weight of about 72,000. At the amino terminus there is a stretch of 12 hydrophobic amino acids, which may represent the signal sequence. Cleavage of this sequence results in a polypeptide with a molecular weight of about 70,500. Two additional hydrophobic sequences are located at positions 447–463 and 627–652. The former is probably involved in the fusion activity, as discussed in Section III,C. The hydrophobic amino acid sequence at the carboxy-terminal end is assumed to function as a membrane anchor, which is followed by a cytoplasmic tail of only three amino acids. While a homology of 30% has been observed between the hemagglutinins of influenza A and B viruses (Krystal et al., 1982), no significant values of homology were found when these glycoproteins were compared with the HEF protein of influenza C virus. The similarity between the HEF sequence and the HA sequence
is restricted to the presence of the three hydrophobic domains mentioned above. Using this criterion, sequence alignments have been reported, with six to nine cysteines being conserved in the glycoproteins of the three types of influenza virus (Nakada et al., 1984; Pfeifer and Compans, 1984). Comparison of the other influenza C proteins with their influenza A and B counterparts also revealed only a very low degree of sequence similarity (Yamashita et al., 1989). Together, the sequence data suggest that influenza A and B viruses are more closely related to one another than they are to influenza C virus.

B. Co- and Posttranslational Modifications

Among the modifications of the HEF polypeptide, gycosylation has been studied in greatest detail. In the presence of the inhibitor tunicamycin the unglycosylated form of the protein is obtained (Nagele, 1983; Hongo et al., 1986a). This finding indicates that the native glycoprotein only contains N-linked oligosaccharides, while O-linked carbohydrate structures are absent. As indicated in Fig. 1, the amino acid sequence contains eight consensus sequences Asn–X–Ser/Thr suitable for the attachment of N-linked oligosaccharides (Nakada et al., 1984; Pfeifer and Compans, 1984). Analysis of the synthesis of the influenza C glycoprotein in the presence of limiting concentrations of glycosylation inhibitors suggested the presence of seven oligosaccharides on the native protein (Nagele, 1983): six on the HEF₁ portion and only one on HEF₂.

Three size classes of oligosaccharides—G₁, G₂, and G₃—have been resolved by gel chromatography (Nakamura et al., 1979). Oligosaccharides corresponding to the two smaller size classes (i.e., G₂ and G₃) have also been observed in influenza A virus, while G₁ is restricted to influenza C virus. G₃ appears to represent the mannose-rich type of oligosaccharides. The oligosaccharides of size classes G₁ and G₂ have both been shown to contain N-acetyleneuraminic acid, indicating that they are of the complex type. Because of the presence of sialic acid on the viral surface, influenza C virus is able to inhibit the hemagglutinating activity of influenza A viruses (Nerome et al., 1976; Meier-Ewert et al., 1978). The structure of the different oligosaccharides has not been determined. It has been suggested that some HEF polypeptides contain predominantly oligosaccharides of the larger size classes, while others are glycosylated with the smaller size classes (Nagele, 1983). This would provide an explanation as to why, after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), HEF is detected as a doublet band (Herrler et al., 1979; Sugawara et al., 1981).
As discussed in Section II,D, glycosylation of HEF is important for the presentation of the antigenic epitopes. Furthermore, the carbohydrate side chains are crucial for the stability of the glycoprotein by protecting it from proteolytic degradation. In the presence of tunicamycin, virions are released from the infected cells; however, the virus particles are lacking surface proteins (Hongo et al., 1986a).

Another posttranslational modification of HEF is the proteolytic cleavage of the precursor polypeptide HEF₀ into the cleavage products HEF₁ and HEF₂. As discussed in Section III,C, this modification is required for viral fusion activity. Cleavage is caused by a cellular protease. Some cultured cells [e.g., chick embryo fibroblasts, LLC-MK2, or Madin–Darby canine kidney (MDCK) cells] are lacking an appropriate enzyme or have only low amounts of it. On the surface of virions released by such cells, the glycoprotein HEF is found predominantly in the uncleaved form, which can be cleaved *in vitro* by incubation with trypsin and elastase (Compans et al., 1977; Herrler et al., 1979; Sugawara et al., 1981). Influenza C viruses grown in embryonated eggs or primary chick kidney cells contain most of their glycoprotein molecules in the cleaved form. The cleavage products are detected after SDS–PAGE only in the presence of reducing agents (Herrler et al., 1979).

This observation indicates that the two polypeptides are held together by disulfide bonds, as observed with several viral surface glycoproteins which are proteolytically cleaved. The disulfide bonds contribute to a unique electrophoretic behavior of HEF which is not observed with the glycoproteins of other influenza viruses. Under nonreducing conditions the electrophoretic mobility of HEF₀ suggests a molecular weight of about 100,000, which is not in accord with the size deduced from the sequence data. In the presence of reducing agents, the electrophoretic migration of the uncleaved glycoprotein suggests a molecular weight of about 80,000, which is in the size range expected for the glycosylated HEF₀. A shift from the 100K form to the 80K form is also observed under nonreducing conditions after proteolytic cleavage of HEF₀ into the disulfide-bonded products HEF₁ and HEF₂.

**Fig. 1.** DNA sequence of gene segment 4 of influenza C/JHB/1/66 and its translation in open reading frame 1 (Pfeifer and Compans, 1984). The sequence is written in message sense. Hydrophobic sequences are marked with wavy lines. The predicted HEF₁–HEF₂ cleavage site is indicated by an arrow. The predicted cleavage site of the leader peptide is indicated by an open triangle. Solid circles indicate potential glycosylation sites. Open circles indicate cysteine residues conserved among hemagglutinins of influenza A, B, and C viruses. The active-site serine (amino acid 71) is indicated by an open square. The mutation site of a mutant with increased receptor-binding efficiency is marked with a solid square (Thr 284).
(designated HEF\textsubscript{1,2}). No evidence for the release of a polypeptide could be obtained, which would explain the shift in the molecular weight by about 20,000 (Meier-Ewert \textit{et al.}, 1980, 1981a,b).

Therefore, it is assumed that the uncleaved glycoprotein has a peculiar conformation which is maintained by disulfide bonds. This conformation may allow only association with a reduced amount of SDS, thereby causing aberrant electrophoretic migration behavior. The conformational constraint is released either by abolishing the disulfide bonds or by proteolytic cleavage of HEF\textsubscript{0} into HEF\textsubscript{1} and HEF\textsubscript{2}. It is not known whether the formation of disulfide bonds is a co- or posttranslational modification of the glycoprotein. The proteolytic cleavage was found to be a late modification. In pulse–chase experiments hardly any cleaved glycoprotein was detectable in infected chick kidney cells. Therefore, the proteolytic cleavage may occur only shortly before virus particles are released by budding.

A modification of the influenza C glycoprotein, which has been described only recently, is the acylation with fatty acids (Veit \textit{et al.}, 1990). The acyl chains are attached presumably to cysteine residues, as indicated by their release after treatment with either hydroxylamine or mercaptoethanol. Such a labile thioester-type linkage has been found on many acylated glycoproteins of both viral and cellular origin. In all cases tested palmitic acid was the predominant fatty acid. The HEF glycoprotein was unique in this respect, because stearic acid was detected as the prevailing fatty acid. The reason for this difference in the acylation is unknown. Cysteine residues in the cytoplasmic tail have been identified as fatty acid attachment sites for several glycoproteins. The cytoplasmic domain of HEF is very short. It contains only a single cysteine, which, therefore, is the candidate for attachment of stearic acid. So far, no biological function can be attributed to the fatty acid of the influenza C glycoprotein.

C. Supramolecular Structure of the HEF Spike

A characteristic feature of influenza C virus was revealed by electron microscopy long before the proteins had been analyzed. The surface projections are usually arranged in a reticular structure consisting mainly of hexagons (Flewett and Apostolov, 1967), which can be seen on both filamentous and spherical particles (Fig. 2). A single spike protein is observed on each of the six vertices of the hexagons (Herrler \textit{et al.}, 1981). Values for the length of individual spikes are in the range of 8–15 nm. The low-resolution structure of the spikes determined by electron microscopy indicated that the influenza C glycoprotein is a trimer (Hewat \textit{et al.}, 1984). The trimeric structure was confirmed when the sedimentation of the glycoprotein in sucrose
Fig. 2. Electron micrograph of influenza C virions. Both a spherical and a filamentous particle are shown. (Adapted from Herrler et al., 1981.)

gradients was analyzed (Formanowski and Meier-Ewert, 1988; Formanowski et al., 1989). Glycoprotein, which has been released from the viral surface by bromelain treatment, is still found as a trimer, indicating that the membrane anchor and the cytoplasmic tail are not essential for maintaining this structure. Calcium ions, however, appear to play an important role. On sucrose gradient centrifugation of bromelain-released HEF, trimers were detected only in the presence of calcium ions. When calcium-deficient buffers were used, the glycoprotein dissociated into monomers (Formanowski and Meier-Ewert, 1988; Formanowski et al., 1989).

Lateral interactions between trimeric spike glycoproteins are probably involved in the formation of the hexagonal array on the viral surface. This is suggested by the finding that the reticular arrangement is sometimes maintained after removal of the spikes from the viral membrane by either protease treatment or spontaneous release (Herrler et al., 1981). Lateral interactions are also suggested by electron micrographs of detergent-isolated spikes. On removal of the detergent, membrane glycoproteins (e.g., the influenza A hemagglutinin) form rosettes, where the proteins are connected at a central point via their hydrophobic membrane anchor. In contrast, the influenza C glycoproteins are arranged in an elongated beetlelike structure with individual spikes standing side by side (Formanowski et al., 1989, 1990). The lateral interactions are not dependent on the proteolytic cleavage of the glycoprotein. The hexagonal array is observed with virus containing predominantly HEF₀ as well as with virus containing HEF₁,₂. Glycoprotein in the uncleaved form also maintains the reticular pattern at pH 5.0. Cleaved glycoprotein, however, undergoes a major conformational change at low pH, resulting in the loss of the
regular hexagonal arrangement of the spikes (Hewat et al., 1984; Formanowski et al., 1990).

It has been reported that crystals of bromelain-released HEF have been obtained, which are suitable for X-ray diffraction studies (Rosenthal et al., 1990). Thus, there is hope that in the near future the three-dimensional structure of the influenza C glycoprotein will be known, which would represent major progress toward understanding the structure–function relationship.

**D. Antigenic Epitopes**

Among monoclonal antibodies directed against the HE protein, two groups have been distinguished. Group A antibodies inhibited the hemagglutinating and hemolytic activities and neutralized the infectivity of influenza C virus, whereas group B antibodies lacked any of these reactivities (Sugawara et al., 1986). Analysis of antigenic variants selected for resistance against either of these monoclonal antibodies suggested the presence of four antigenic epitopes: A-1, A-2, B-1, and B-2 (Sugawara et al., 1988). Competitive binding assays indicated that sites A-1 and A-2 may be located close to one another (Sugawara et al., 1988). Both A epitopes were shown to be sensitive to denaturing conditions (e.g., treatment with SDS). Therefore, on Western blots, HEF was detected only by group B antibodies. These results indicate that sites B-1 and B-2 are sequence-dependent epitopes, whereas sites A-1 and A-2 are conformation-dependent epitopes. The conformation of both A epitopes was found to be dependent on the glycosylation of HEF. The nonglycosylated form of the protein synthesized in the presence of tunicamycin was recognized by group B antibodies, while group A antibodies reacted only poorly or not at all (Hongo et al., 1986; Sugawara et al., 1988). The antigenic sites are presumably different from the functional epitopes of the receptor-binding and receptor-destroying activities. The ability of several monoclonal antibodies to inhibit the hemagglutinating activity of influenza C virus (Sugawara et al., 1986, 1988; Vlasak et al., 1987; Herrler et al., 1988a) may be due to steric hindrance. Some of the antibodies caused partial inhibition of the receptor-destroying enzyme, when the esterase activity was determined with large substrates, but no inhibitory effect was observed when small substrates were used (Vlasak et al., 1987; Herrler et al., 1988a; Hachinohe et al., 1989).

The antigenic variation among different strains of influenza C virus is less pronounced than in the case of influenza A viruses. Several reports revealed a high degree of cross-reactivity between different strains, irrespective of the time and place of isolation (Czekalowski and Prasad, 1973; Chakraverty, 1974, 1978; Meier-Ewert et al., 1981c;
Kawamura et al., 1986). Using monoclonal antibodies, it was possible, however, to demonstrate antigenic variation (Sugawara et al., 1986, 1988; Adachi et al., 1989). The low extent of variation is not due to a low capacity to produce antigenic variants. Escape mutants resistant against monoclonal antibodies have been obtained with a frequency similar to values reported for influenza A virus (Sugawara et al., 1988). Maybe the immune selection is less pronounced in the case of influenza C virus. This may also explain why no antigenic drift has been observed with this group of viruses. Among the antigenic variants arising within influenza A viruses, one usually becomes dominant and replaces the older ones. In contrast, analyses of different influenza C strains indicate that several antigenic variants cocirculate (Adachi et al., 1989). This conclusion is supported by studies on the genetic variation in the HEF as well as the NS gene of influenza C virus (Buonagurio et al., 1985, 1986; Kawamura et al., 1986; Adachi et al., 1989).

III. Functions

A. Receptor-Destroying Enzyme

Although the receptor-destroying enzyme of influenza C virus was described by Hirst in 1950, more than 30 years passed before its specificity was elucidated. Identification of the enzyme activity was accomplished by analyzing its effect on hemagglutination inhibitors. Rat serum has long been known for its inhibitory activity (Styk, 1955; O'Callaghan et al., 1980). Two components of rat serum have been shown to account for most of the hemagglutination inhibition activity: murinoglobulin and $\alpha_1$-macroglobulin (Herrler et al., 1985b; Kitame et al., 1985). The carbohydrate portion of the latter compound was found to consist primarily of N-linked biantennary oligosaccharides (Herrler et al., 1985b). The only effect of the influenza C enzyme on these oligosaccharides was a change in the terminal sialic acid residue. While the native macroglobulin contained 40% of its sialic acid as N-acetyl-9-O-acetylneuraminic acid, this amount was reduced to 10% after treatment with the receptor-destroying enzyme.

Concomitant with the decrease of the 9-O-acetylated sialic acid, an increase of N-acetylneuraminic acid was observed (Herrler et al., 1985c). The same effect was obtained with bovine submandibulary mucin, another hemagglutination inhibitor. In both cases the change in the sialic acid was paralleled by loss of the inhibitory activity, indicating that the receptor-destroying enzyme of influenza C virus is a sialate 9-O-acetylesterase (Fig. 3). The enzyme has been shown to be
Neuraminide \(\beta\)-acetylesterase

\[
\begin{array}{c}
\text{Neuraminidase} \\
\text{H}_3C\text{O} - \text{C} - \text{O} - \text{C} - \text{H} - \text{C} - \text{OH} - \text{H} - \text{OH} - \text{N} - \text{C} - \text{O} - \text{OH} - \text{CH}3
\end{array}
\]

**Fig. 3.** Structure of \(N\text{-acetyl-9-O-acetylneuraminic acid connected to galactose via an }\alpha2,3\text{-linkage. The sites of action of the acetylesterase of influenza C virus and the neuraminidase of influenza A and B viruses are shown.}**

a function of HEF by several approaches: expression of the cloned HEF gene in vertebrate cells (Vlasak et al., 1987) and analysis of the purified protein after isolation by detergent (Herrler et al., 1988a) or protease treatment (Formanowski and Meier-Ewert, 1988).

The influenza C esterase belongs to the class of serine hydrolases which are inhibited by diisopropyl fluorophosphate (DFP). The inhibitor abolishes the enzyme activity without affecting the hemagglutinating activity (Muchmore and Varki, 1987). This finding suggests that the active site of the esterase and the receptor-binding site are different epitopes on the influenza C glycoprotein. There is some information on the amino acids which are crucial for the formation of the active site of the esterase. From the knowledge about other serine hydrolases (e.g., trypsin and chymotrypsin), it is expected that the enzyme mechanism involves a charge relay system, which is accomplished by a catalytic triad composed of the amino acids serine, histidine, and aspartic acid (Kraut, 1977). Taking advantage of the fact that DFP binds covalently to the serine in the active site of serine hydrolases, amino acid 71 of HEF has been identified as active-site serine (Herrler et al., 1988b; Vlasak et al., 1989). This amino acid is part of the sequence Phe–Gly–Asp–Ser–Arg (Fig. 1). While the motif Gly–Asp–Ser is found in the active site of many serine hydrolases, including trypsin and chymotrypsin, the following arginine residue has been detected so far only in the active site of the acetylerases of influenza C virus and coronaviruses (see Section IV and Fig. 5). From inhibition studies with arginine-specific modifying reagents, it has been suggested that this arginine residue may be important for substrate recognition, possibly interacting with the carboxyl group of \(N\text{-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac2) (Hayes and Varki, 1989).}**

Analysis of a series of compounds revealed that the esterase of influenza C virus has a high specificity for O-acetyl groups, Neu5,9Ac\(_2\)
TABLE I

| Substrate                                                                 | Relative cleavage rate (%) |
|--------------------------------------------------------------------------|----------------------------|
| N-Acetyl-9-O-acetylneuraminic acid                                       | 100                        |
| N-Acetyl-4-O-acetylneuraminic acid                                       | 3                          |
| N-Glycolyl-9-O-acetylneuraminic acid                                     | 33                         |
| N-Acetyl-7-O-acetylneuraminic acid                                       | —                          |
| Bovine submandibular gland mucin                                         | 30                         |
| Rat serum glycoprotein                                                   | 90                         |
| Rat erythrocytes                                                         | 25                         |
| Equine submandibular gland mucin                                         | —                          |
| 4-Methylumbelliferyl acetate                                             | 220                        |
| 4-Methylumbelliferyl butyrate                                            | 14                         |
| 4-Nitrophenyl acetate                                                    | 3500                       |
| \(\alpha\)-Naphthyl acetate                                             | 2200                       |

*From Schauer et al. (1988).*

being hydrolyzed at the highest rate among all natural substrates tested (Schauer et al., 1988) (see Table I). Some aromatic acetates (e.g., 4-nitrophenyl acetate or \(\alpha\)-naphthyl acetate) are cleaved at higher rates than Neu5,9Ac2 (Vlasak et al., 1987; Schauer et al., 1988; Wagaman et al., 1989). These compounds are substrates for many serine hydrolases, including proteases, and therefore are not suited for determination of the enzyme specificity. They enable, however, fast and sensitive assays. \(\alpha\)-Naphthyl acetate has been shown to be useful for cytochemical detection of influenza C-infected cells (Wagaman et al., 1989). Treatment of erythrocytes with influenza C virus has been reported to change the reactivity of the cells with lectins specific for N-acetylgalactosamine (Luther et al., 1988). From this finding it has been inferred that the receptor-destroying enzyme is able to release the acetyl residue of N-acetylgalactosamine. However, there is no direct chemical evidence supporting this conclusion.

Apart from DFP the esterase activity of influenza C virus is also inhibited by diethyl-4-nitrophenyl phosphate and some isocoumarins (Schauer et al., 1988; Vlasak et al., 1989). Inhibition of the esterase by DFP or isocoumarins has been reported to reduce the infectivity of the virus (Muchmore and Varki, 1987; Vlasak et al., 1989). This finding may suggest that the receptor-destroying enzyme is required for virus entry into cells. However, both the hemagglutination (i.e., receptor-binding) and hemolytic (i.e., fusion) activities are not affected by the inactivation of the esterase (Muchmore and Varki, 1987; Vlasak et al., 1989). Thus, more experiments are necessary to show whether the
TABLE II

Receptor Specificity of Influenza A, B, and C Viruses

| Sialic acid on human erythrocytes | HA titer (HA units/ml) | C/JHB/1/66 | B/HK/8/73 | A/PR/8/34 |
|----------------------------------|------------------------|------------|-----------|-----------|
| Native                           | 0                      | 64         | 256       |           |
| Asialo                           | 0                      | 0          | 0         | 0         |
| Neu5Ac                           |                        |            |           |           |
| $\alpha_{2,3}Gal\beta_1,3GalNAc$ | 0                      | 2          | 256       |           |
| $\alpha_{2,3}Gal\beta_1,4GlcNAc$ | 0                      | 128        | 128       | 128       |
| $\alpha_{2,6}Gal\beta_1,4GlcNAc$ | 0                      | 64         | 128       | 128       |
| Neu5Gc                           |                        |            |           |           |
| $\alpha_{2,6}Gal\beta_1,4GlcNAc$ | 0                      | 2          | 0         | 0         |
| Neu5,9Ac$_2$                     |                        |            |           |           |
| $\alpha_{2,3}Gal\beta_1,3GalNAc$ | 128                    | 0          | 0         | 0         |
| $\alpha_{2,3}Gal\beta_1,4GlcNAc$ | 128                    | 0          | 0         | 0         |
| $\alpha_{2,6}Gal\beta_1,4GlcNAc$ | 128                    | 0          | 0         | 0         |

$^a$ Adapted from Rogers et al. (1986).

$^b$ 0, HA titer <2.

reduction of the infectivity is correlated with the inhibition of the enzyme activity or whether it is due to an indirect effect of the inhibitor.

B. Receptor-Binding Activity

The identification of the receptor-destroying enzyme as a sialate 9-$O$-acetylesterase implied that Neu5,9Ac$_2$ (see Fig. 3) is a crucial component of the cellular receptors for influenza C virus (Herrler et al., 1985). Direct evidence for the role of Neu5,9Ac$_2$ as a receptor determinant was provided by studies with erythrocytes which had been modified to contain only a single type of sialic acid. Influenza C virus was able to agglutinate erythrocytes which had been sialylated with Neu5,9Ac$_2$, but not cells containing $N$-acetyl- or $N$-glycolylnueuraminic acid (Rogers et al., 1986) (see Table II). On the basis of these results, it was possible to explain previous observations which seemed to argue against an involvement of sialic acid in the attachment of influenza C virus to cells. The resistance of the erythrocyte receptors to periodate treatment (Ohuchi et al., 1978) is due to a greatly reduced oxidation of Neu5,9Ac$_2$ by periodate compared to Neu5Ac (Haverkamp et al., 1975). The difficulty in inactivating the influenza C receptors with viral and bacterial neuraminidases (Hirst, 1950; Kendal, 1975; Herrler et al., 1985) is explained by the relative resistance of Neu5,9Ac$_2$ to the action of these enzymes (Corfield et al., 1981). The importance of
Neu5,9Ac2 as a receptor determinant is not restricted to erythrocytes. 9-O-Acetylated sialic acid is also required for influenza C virus to initiate the infection of cultured cells (Herrler and Klenk, 1987a). In fact, lack of this type of sialic acid is a major reason for the resistance of many cell lines to influenza C virus.

Insertion of artificial receptors into the plasma membrane of cultured cells rendered several resistant cells sensitive to an influenza C infection. Moreover, an increase in the yield of virus was observed with cells which usually produce only low amounts of virus (Herrler and Klenk, 1987a). The presence of 9-O-acetylated sialic acid appears to be the major factor in determining whether a glycoconjugate can serve as a receptor for influenza C virus. Erythrocytes which have been resialylated to contain Neu5,9Ac2 were agglutinated by influenza C virus regardless of whether the sialic acid molecule was attached to galactose via an α-2,3 or α-2,6 linkage (Rogers et al., 1986) (see Table II). The sialyltransferase specific for the latter linkage type only acts on glycoproteins. Therefore, receptors generated by this enzyme are glycoproteins. On the other hand, it has been shown that bovine brain gangliosides can also serve as receptors for influenza C virus, although the active species among the mixture of gangliosides has not been determined (Herrler and Klenk, 1987a,b). Thus, both glycoproteins and glycolipids can be used as receptors by influenza C virus, provided they contain Neu5,9Ac2. A larger number of glycoconjugates must be analyzed, however, in order to know whether factors other than the presence of Neu5,9Ac2 are important for the receptor function of a glycoconjugate.

It has been suggested that, in addition to Neu5,9Ac2, influenza C virus may also recognize N-acetylgalactosamine (Luther et al., 1988). The conclusion is based on the finding (mentioned in Section III,A) that erythrocytes treated with influenza C virus differ from control cells in their reactivity with lectins specific for N-acetylgalactosamine. However, direct evidence for such a receptor specificity is lacking.

The amino acids involved in the receptor-binding site of HEF have not been determined. Valuable information should be obtained by the analysis of mutants with a change in the receptor-binding activity. A mutant has been described which has an expanded cell tropism due to a more efficient recognition of Neu5,9Ac2-containing receptors compared to the parent virus (Szepanski et al., 1989). Sequence analysis of this mutant indicated that a single point mutation (Thr 284 to isoleucine; see Fig. 1) is responsible for the change in the receptor-binding activity (Szepanski et al., 1991). Interestingly, the mutation site is located next to a sequence (Gly–Asn–Ser–Gly) which, in similar form
(Gly–Gln–Ser–Gly), is also found in several subtypes of influenza A hemagglutinins (Fig. 1). The homologous sequence in the H3 subtype composing amino acids 225–228 has been shown to be part of the receptor-binding pocket (Weis et al., 1988). These data suggest that the amino acids Gly 279 to Thr 284 may be constituents of the receptor-binding site of HEF and that the mutant is altered at this site. The observation that these amino acids are located on the unfolded polypeptide at a distance far from Ser 71 at the catalytic center of the esterase, together with the DFP effects (Section III,A), supports the notion that receptor binding and receptor inactivation are exerted by different structural domains of HEF.

Another example of an influenza C virus with a change in the receptor-binding activity has been reported (Camilleri and Maassab, 1988). Virus isolated from persistently infected MDCK cells was found to be more sensitive to the action of hemagglutination inhibitors than was wild-type virus. Sequence analysis of more mutants or variants of this type should help further define the receptor-destroying and receptor-binding sites of HEF. Obviously, however, final answers to these problems can be given only when the three-dimensional structure of the glycoprotein is available. The importance of individual amino acids involved in the formation of the functional epitopes can then be evaluated by site-directed mutagenesis.

The ability of the influenza C glycoprotein to attach to Neu5,9Ac2-containing receptors can be used as a powerful tool to detect 9-O-acetylated sialic acid (Muchmore and Varki, 1987). The ability of influenza C virus to agglutinate erythrocytes from an adult chicken, but not those from a 1-day-old chicken, was the basis for the discovery that Neu5,9Ac2 is a differentiation marker on chicken erythrocytes, which has been confirmed by chemical analysis of the sialic acids on these cells (Herrler et al., 1987). The sensitivity of the receptor recognition by influenza C virus is evident from studies with human erythrocytes. By chemical analysis only Neu5Ac has been detected, not Neu5,9Ac2 (Shukla and Schauer, 1982). Agglutination and binding studies indicate, however, that erythrocytes from some individuals contain low levels of 9-O-acetylated sialic acid on their surface (Ohuchi et al., 1978; Nishimura et al., 1988).

C. Membrane Fusion

The fusion activity of influenza C virus was first demonstrated with erythrocytes. Microscopic observation of virus-induced cell fusion and photometric detection of hemolysis indicated that the virus is able to fuse with mouse and chicken erythrocytes (Ohuchi et al., 1982; Kitame et al., 1982). Recently, the fusion between virus membranes and ar-
HEF GLYCOPROTEIN OF INFLUENZA C VIRUS 227
tificial membranes has been monitored using a resonance energy as-
say (Formanowski et al., 1990). In contrast to the hemagglutinating
(Herrler et al., 1979; Sugawara et al., 1981) and esterase activities of
HEF (Herrler et al., 1988a), the fusion activity requires the proteolytic
cleavage of HEF$_0$ into polypeptides HEF$_1$ and HEF$_2$ (Ohuchi et al.,
1982; Kitame et al., 1982), described in Section II,B. The dependence
of the influenza C virus-induced fusion on the cleavage of HEF indicated
that this activity is a function of the surface glycoprotein. Virus with
uncleaved glycoprotein can be rendered fusigenic by in vitro cleavage
of HEF. Both trypsin and elastase have been shown to be effective in
this respect, whereas other proteases (e.g., chymotrypsin and ther-
molysin) were unable to activate the glycoprotein (Kitame et al.,
1982; Ohuchi et al., 1982; Formanowski et al., 1990).

An additional characteristic of the fusion activity is pH dependence.
Similar to influenza A and B viruses and several other viruses, influ-
enza C virus causes fusion only at a low pH. Optimal pH values for
hemolysis of erythrocytes vary between 5.0 and 5.7, depending on the
virus strain. Optimal fusion between virus and unilamellar liposomes
was detected in the range of 5.6–6.1. Several changes have been ob-
served when the glycoprotein is shifted from neutral to acidic pH val-
ues: (1) The glycoprotein becomes susceptible to trypsin digestion;
(2) the endogenous tryptophan fluorescence decreases; and (3) the hex-
agonal arrangement of the surface projections disappears (For-
manowski et al., 1990). These changes, which were only observed with
virus containing the cleaved HEF (i.e., HEF$_{1,2}$), suggest that exposure
to a low pH results in a conformational change of the glycoprotein.

The characteristics of the influenza C virus-induced fusion de-
scribed so far (i.e., a dependence on both proteolytic cleavage and low
pH and a conformational change at low pH) are very similar to those
reported for the fusion activity of influenza A and B viruses. It is
therefore likely that fusion occurs by a similar mechanism for all
influenza viruses. With influenza A virus it is widely accepted that the
conformational change observed at acidic pH results in the exposure of
the amino-terminal portion of the membrane-bound cleavage product
(HA$_2$). This part of the protein is made up of a stretch of hydrophobic
amino acids, which probably interact with the cellular membrane,
thereby inducing fusion between the viral envelope and the membrane
of the target cell. This model is also applicable to influenza C virus.

Differences between influenza A and C viruses have been observed so
far as the kinetics of the fusion process are concerned. In the case of
influenza A virus, the conformational change is fast and a later step is
rate limiting. With influenza C virus the conformational change has
been found to be a rate-limiting step (Formanowski et al., 1990). The
reason for the delayed conformational change may be related to the
hexagonal arrangement of the spikes. The close packing of the glycoproteins might be a hindrance in adopting the conformation required for fusion.

In the course of virus infection, the viral fusion activity is crucial for the penetration of enveloped viruses. Viruses with a pH-dependent fusion activity are generally assumed to enter a cell via endosomes. The acidic pH within such vesicles triggers the fusion reaction, resulting in the release of the nucleocapsid into the cytoplasm. This may also apply to influenza C virus, although no evidence has been presented to support this assumption. In any case the fusion activity is crucial for the infectivity of the virus. Virus with uncleaved glycoprotein is lacking not only fusion activity, but also infectivity (Herrler et al., 1979; Sugawara et al., 1981). Restoration of the fusion activity in vitro by proteolytic cleavage of the glycoprotein is accompanied by restoration of the infectivity.

Due to the characteristics of the glycoprotein HEF, influenza C virus is unique among myxoviruses. Influenza A and B viruses as well as paramyxoviruses differ from influenza C virus in the specificity of the receptor-binding activity (Neu5Ac versus Neu5,9Ac₂) and the receptor-destroying enzyme (neuraminidase versus acetyl esterase). In addition, HEF is responsible for three activities (receptor binding, receptor inactivation, and fusion), while both paramyxoviruses and influenza A and B viruses have two surface glycoproteins for these activities (Fig. 4). The unique characteristics of the influenza C glycoprotein are reflected in the designation “HEF,” which has been proposed to indicate that this protein can function as a hemagglutinin,
as an esterase, and as fusion factor (Herrler et al., 1988a). Others have chosen the designation "HE" (Vlasak et al., 1987), which ignores the fusion activity. In addition, there is an HE protein present on some coronaviruses (Cavanagh et al., 1990). This protein, described in Section IV, has hemagglutinating and esterase activities, but no fusion activity. Thus, "HEF" is an appropriate designation for the influenza C glycoprotein, to distinguish it from the coronavirus glycoprotein.

IV. RELATIONSHIP BETWEEN HEF AND THE CORONAVIRUS GLYCOPROTEIN HE

For many years ortho- and paramyxoviruses have been thought to be the only animal viruses containing receptor-destroying enzymes. Prompted by a sequence similarity between an open reading frame on the genome of mouse hepatitis virus and the HEF gene of influenza C virus (Luiftjes et al., 1988) it was found that bovine coronavirus (BCV) is able to inactivate its own receptors on erythrocytes (Vlasak et al., 1988a). The enzyme turned out to be a sialate 9-O-acetyesterase similar to the receptor-destroying enzyme of influenza C virus. In fact, the coronavirus enzyme was able to inactivate the receptors for influenza C virus on erythrocytes, and the esterase of influenza C virus inactivated the receptors for BCV (Vlasak et al., 1988a), indicating that both viruses use the same receptor determinant for attachment to cells (i.e., Neu5,9Ac2). This conclusion was confirmed by resialylation studies with erythrocytes and has been extended to a porcine coronavirus, hemagglutinating encephalomyelitis virus (HEV) (Schultze et al., 1990).

An acetyesterase activity has been reported not only for BCV but also for HEV and some strains of mouse hepatitis virus (Yokomori et al., 1989; Schultze et al., 1991a; Pfleiderer et al., 1991). The acetyesterase activity of BCV has been shown to be a function of a surface glycoprotein which is detected as a disulfide-linked dimer with a molecular weight of about 140,000. (Vlasak et al., 1988b; Schultze et al., 1991a). The same protein has been identified previously as a hemagglutinin (King et al., 1985), and therefore the designation "HE" has been chosen to indicate its dual function as hemagglutinin and esterase (Cavanagh et al., 1990). Similar to its influenza C counterpart, the esterase of coronaviruses is a serine esterase which can be inhibited by DFP (Vlasak et al., 1988b; Schultze et al., 1990, 1991a). Inhibition of the enzyme activity results in a dramatic reduction of infectivity, suggesting an important role for the esterase in an early stage of the infection (Vlasak et al., 1988b). The importance of the HE protein has also been demonstrated with monoclonal antibodies which
FIG. 5. Alignment of the amino acid sequences of the HEF protein of influenza C/JHB/1/66 (1) and the HE protein of mouse hepatitis virus, strain JHM (2). Every tenth position is indicated by a dot. Wavy lines indicate hydrophobic sequences. With HEF these compose the amino-terminal signal sequence (amino acids 1-12), the presumptive fusion peptide (amino acids 447-463), and the membrane anchor (amino acids 624-652). HE has only two hydrophobic regions, the signal peptide (amino acids 1-17) and the membrane anchor (amino acids 404-429), which do not align with the corresponding domains of HEF. The active-site serine of the acetylesterase, which is conserved in both proteins, is marked with an open triangle. Stretches of amino acids identical in both sequences are indicated by underscoring. Vertical lines indicate identical or related amino acids. The figure is based on an alignment of HEFl and HE.

(Courtesy of S. G. Siddell.)
were shown to neutralize BCV both in vivo and in vitro (Deregt et al., 1989).

When the amino acid sequence of the HE protein is aligned with the sequence of the influenza C glycoprotein HEF, homology is observed only with the HEF₁ cleavage product. There is no sequence on the HE protein which is related to the HEF₂ polypeptide (Fig. 5). This observation is not surprising, because HEF₂ is responsible for the fusion activity of influenza C virus, whereas, in the case of coronaviruses, fusion is a function not of HE, but of the S protein (reviewed by Spaan et al., 1988). The homology between the amino acid sequences of HE and HEF₁ has been reported to be 30% (Luytjes et al., 1988). The alignment indicates that there are many conservative substitutions. A few regions are completely identical in both sequences (Fig. 5). Among these is the sequence Phe–Gly–Asp–Ser–Arg, which, in the case of influenza C virus, has been shown to contain the active-site serine of the esterase (Herrler et al., 1988; Vlasak et al., 1989).

It is interesting to note that, on the other hand, the putative constituent sequence of the HEF receptor-binding site (Gly 279–Thr 284) does not have a homologous counterpart in the HE sequence. This observation may be related to the recent finding that HE is not very efficient in agglutinating erythrocytes (Schultze et al., 1991a) and that the major hemagglutinin of BCV is the peplomer glycoprotein S (Schultze et al., 1991b). It has been argued that the extent of identity between HE and HEF₁ is high enough to rule out convergent evolution, and, therefore, it has been speculated that coronaviruses acquired the HE gene from influenza C virus by nonhomologous recombination between ancestors of both viruses (Luytjes et al., 1988). However, acetylesterases are also found in cells. If coronaviruses actually acquired the esterase gene by a recombination event, the gene might as well be derived from a cellular gene. More information about the viral and cellular esterases is required to distinguish between these possibilities.

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