Significance of Viral Glycoproteins for Infectivity and Pathogenicity*

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Abstract

Disease resulting from virus infection is a complex event depending on the close interaction of viral and cellular factors. Through the application of biochemical and genetic methods, it is now possible to gain an insight into the molecular basis of these interactions. Thus, it has been shown that the glycoproteins of enveloped viruses play a central role in the initiation of infection. They are responsible not only for the adsorption of virions to cellular receptors, but are also for the entry of the genome into the cell by the fusion of viral envelopes with cellular membranes. Evidence is growing that the fusogenic glycoproteins are frequently activated by cellular proteases. The structure of the proteins at the cleavage site and the availability of a suitable protease are critical for tissue tropism, spread of the virus in the infected organism and, thus, for pathogenicity. This will be demonstrated here by the example of the haemagglutinin of influenza viruses.

Zusammenfassung

Das Entstehen einer virusbedingten Infektionskrankheit ist ein vielschichtiges Ereignis, das auf einer engen Wechselwirkung viraler und zellulärer Faktoren beruht. Mit Hilfe biochemischer und genetischer Methoden konnten erste Einblicke in die molekularen Grundlagen dieser Zusammenhänge erhalten werden. So hat es sich gezeigt, daß den Glycoproteinen der umhüllten Viren eine zentrale Funktion bei der Initiation der Infektion zukommt. Sie sind verantwortlich für die Adsorption der Viruspartikel an zelluläre Rezeptoren und für das Einschleusen des Virusgenoms in das Zellinnere. Beim Penetrationsvorgang, der auf einer Fusion zwischen der Virusaußenhülle und der Zellmembran beruht, wirken sie als Fusogen. Die meisten bis jetzt näher untersuchten fusogenen Glycoproteine werden durch zelluläre Proteasen aktiviert. Die Struktur der Proteine an der Spaltstelle und das Vorhandensein einer geeigneten Protease sind entscheidende Determinanten für den Gewebstropismus, die Ausbreitung des Virus im infizierten Organismus und damit für die Pathogenität. Am Beispiel des Hämagglutinins der Influenzaviren werden diese Zusammenhänge aufgezeigt.

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A number of viruses possess in addition to the minimal requirement of viruses, the genomic nucleic acid and its surrounding capsid, an envelope, which represents a virus-specific altered cell membrane. It is taken up during virus maturation in the so-called budding process as an integral and essential constituent of the virus particle. As with cellular membranes the envelope consists of a lipid bilayer in which nearly all of the cellular proteins are exchanged for virus-specific glycoproteins, which appear in the various types of enveloped viruses as protrusions of differing structures and morphology. Additionally, in some enveloped viruses, the inner side of the lipid bilayer is covered by a non-glycosylated membrane or matrix (M) protein (for literature see *Rott* and *Klenk*, 1977; *Klenk* and *Rott*, 1980).

The viral glycoproteins possess similar biological functions. As surface components of the virus they represent those antigens against which the immune response of the organism is mainly directed, inducing the B- and T-cell reactivities necessary for protection. These well known properties of the viral glycoproteins, which are already utilized in the development of subunit vaccines, are not the subject of this review. This contribution will, however, concentrate on the role of the glycoproteins in the initiation of infection, making these molecules the determinants of pathogenicity.

Biosynthesis of viral glycoproteins

Like cellular membrane proteins, the viral glycoproteins are synthesized on membrane-bound polyribosomes. The translocation of the growing polypeptide chain through the membrane of the rough endoplasmic reticulum is made possible by the presence of a signal peptide on the amino terminus of the molecule. The signal peptide is eliminated by cotranslational proteolytic cleavage. Likewise the first step of glycosylation occurs cotranslationally, at least with those glycoproteins containing N-glycosidically linked carbohydrate side chains. In this process mannose-rich oligosaccharides are assembled on a dolichol pyrophosphate carrier, and then transferred en bloc to the nascent polypeptide chain (for literature see *Schwarz* and *Datema*, 1982).

The glycoproteins are further modified during transport from their site of synthesis to the site of virus maturation. These posttranslational modifications include the processing of the carbohydrate side chains and a further limited proteolytic cleavage. Glycosylation and posttranslational proteolytic cleavage are, for most viral glycoproteins, essential for the expression of their biological activity. In addition to this some glycoproteins are further modified by sulfatation and fatty acid acylation, the significance of which is not yet fully understood (*Klenk* and *Rott*, 1980; *Schmidt*, 1983). Little is also known about the cellular transport of glycoproteins. Furthermore, it is still an open question as to how the budding process, which leads to the release of newly synthesized virus particles, is triggered.

An important function of the viral glycoproteins is their ability to initiate the processes of infection. Firstly, they are responsible for attachment of viral particles to specific receptors of the host cell surface, and secondly they induce the penetration of the virus genome into the cell. This latter step involves fusion of the virus envelope with the cellular membrane (see *Klenk* and *Rott*, 1980) and appears to be the simplest way for the virus genome to pass through the barrier of two membranes.

Depending upon the virus family, one or two glycoproteins are involved in the adsorption and penetration of enveloped viruses. The mechanisms underlying these processes have been most intensively studied with influenza A viruses. The knowledge

gained from these studies will be therefore discussed in greater depth in the following sections.

Structure of the haemagglutinin of influenza viruses

Influenza A viruses contain on their surfaces two different types of glycoproteins, the haemagglutinin and the neuraminidase. For a number of influenza virus subtypes the amino acid sequences of the haemagglutinins have been elucidated (Literature see *Lamb*, 1983) and the tertiary structure has been determined by X-ray crystallography (*Wilson* et al., 1981). It was shown that the haemagglutinin is a trimer consisting of three identical monomers. Each monomer is comprised of two polypeptides, HA_1 and HA_2, held together by disulfide bonds. HA_1 and HA_2 are derived by posttranslational proteolytic cleavage of a precursor molecule HA. Each polypeptide comprises two structurally distinct regions. HA_2 forms a triple-stranded coiled-coil of alpha helices extending 76 Å from the membrane. The amino terminus of the HA_2 exposed by the proteolytic cleavage has a highly conserved hydrophobic amino acid sequence. The larger polypeptide HA_1 forms a globular domain of anti-parallel β-sheets, which is positioned on top of the HA_2 stem. It contains the variable antigenic determinants responsible for the characteristic recurrence of influenza infections in man, and the receptor binding site of the molecule (Literature see *Skehel* et al., 1984). Each polypeptide has an unusual loop-like topology, which starts at the membrane, extends 135 Å distally and then folds back to enter the envelope. Both complex and mannose-rich carbohydrate side chains are present on the surface of the trimer, with only the latter type being specifically located in niches at the interfaces between different domains (*Keil* et al., 1984).

Proteolytic activation of influenza virus haemagglutinins

During infection the influenza virus particles are adsorbed to neuraminic acid containing receptors of the host cell through the receptor binding site of the haemagglutinin. Under acidic conditions a change in the conformation of the molecule occurs (*Skehel* et al., 1982), through which the hydrophobic region at the amino terminus of HA_2, the so-called fusion peptide, becomes exposed. This leads, possibly via a still hypothetical secondary receptor, to close contact between the lipid lamellae of the virus and cellular membranes, resulting in membrane fusion. The secondary receptor appears to become available in some instances through the action of the virus neuraminidase (*Huang* et al., 1981). Since the fusion peptide becomes exposed after the posttranslational proteolytic cleavage of the haemagglutinin, the fusion capacity of the molecule is activated only by this process. Cleavage, therefore, is an essential condition for the infectivity of the virus (*Klenk* et al., 1975; *Lazarowitz* and *Choppin*, 1975).

The influenza virus haemagglutinin is activated in the same way as a number of precursors of enzymes and hormones, e.g. proinsulin, progastrin and proopiomelanocortin (for literature see *Docherty* and *Steiner*, 1982). In all these cases trypsin-like endoproteases attack the precursor at a given cleavage site behind an arginine. This is followed by carboxypeptidase cleavage, which eliminates the peptide linking the two polypeptides (*Garten* and *Klenk*, 1983).

Since the activating proteases are cellular enzymes cleavage of the haemagglutinin depends strictly on the type of host cell. Virus particles, which have been produced in a host cell deficient in activating proteases and which have an uncleaved haemagglutinin and are non-infectious can be transformed into the infectious form by *in vitro* treatment with appropriate enzymes (*Klenk* et al., 1975; *Lazarowitz* and *Choppin*, 1975). Microorganisms may represent one source of enzymes capable of proteolytic activation (see below).

Influenza virus haemagglutinins as a determinant of pathogenicity

Haemagglutinins from different strains of influenza viruses differ in their sensitivity to proteolytic cleavage in a variety of host cells. This correlates with the spread of the virus in the host organism. Influenza viruses have been isolated from man, horse, swine, mink, seal and whale as well as from a great variety of different avian species. The infection in mammals is usually confined to the respiratory tract, while in birds, there is a systemic infection which has been denominated by the suffix "pest". Mammalian viruses have always been isolated from individuals with clinical manifestations and the isolated virus has usually been pathogenic. Most avian viruses, on the other hand, do not cause any, or cause only, mild signs of disease with local infections of the respiratory or intestinal tract being clinically inapparent. All highly pathogenic avian influenza viruses belong to the subtypes H5 and H7 (*Rott* and *Klenk*, 1987).

Whereas the haemagglutinins of influenza viruses of mammals and birds which cause a local infection are only cleaved in a few special types of cells, the haemagglutinins of the pathogenic avian influenza viruses are activated by proteases occurring in all types of cells examined so far (*Klenk* et al., 1975; *Rott* et al., 1980). Even insect cells possess an enzyme capable of activating the haemagglutinins of these pathogenic avian influenza viruses (*Kuroda* et al., 1986). Therefore, there are considerable differences in the host spectrum of these viruses which can be traced back to the different cleavabilities of their haemagglutinins.
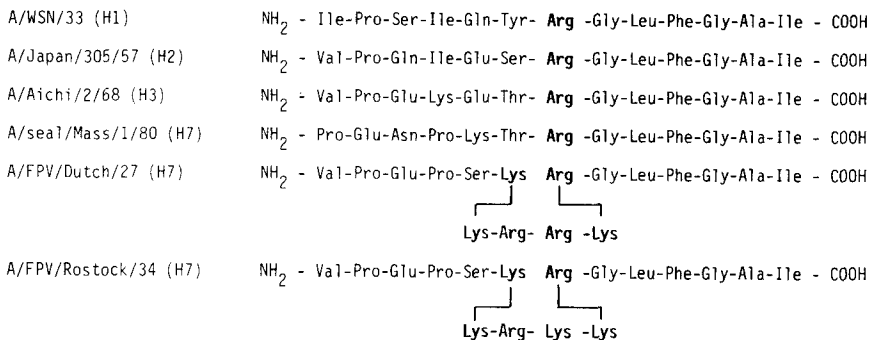

Fig. 1. Influenza viruses, which cause a local infecton, have the HA_1 and HA_2 polypeptides of the haemagglutinin linked by a single arginine residue, whereas in the pathogenic avian influenza viruses the intervening peptide consists of several basic amino acids (for literature see *Rott* and *Klenk*, 1986).

The differences in cleavability are based on structural differences in the cleavage site of the haemagglutinins (Fig. 1). Sequence analyses have shown that in the haemagglutinins of the mammalian viruses and those of the apathogenic avian influenza viruses, the polypeptides HA_1 and HA_2 are linked through a single arginine residue. On the other hand, the haemagglutinins of the pathogenic avian influenza viruses have an intervening sequence of several basic amino acids between HA_1 and HA_2 in which the sequences lysine–arginine or arginine–arginine regularly appear (see *Rott* and *Klenk*, 1986). Although the haemagglutinins of all influenza viruses are cleaved by the same general mechanism, the available data indicate that differences in specificity of the proteases exist, such that either a single arginine or the basic pairs, lysine–arginine and arginine–arginine, can be recognized and eliminated by the cleavage reaction. The differences in the manifestation of infection are therefore due to the structural differences of the haemagglutinins alone (*Bosch* et al., 1981; *Garten* et al., 1981). The structural differences, upon which the different proteolytic activities depend, are restricted therefore to a small, but functionally important part of the molecule (*Rott and Klenk*, 1986). The haemagglutinin therefore becomes a critical determinant of pathogenicity.

Alterations in cleavability through mutation

It is therefore not surprising that the substitution of a single amino acid in the cleavage site can influence the cleavability of the haemagglutinin and as a result the pathogenicity of the virus. In addition to mutations in the cleavage site itself, a mutation in close proximity to the cleavage site can also affect the activation of the molecule. For instance, after adaptation of an influenza virus to MDCK cells, the protease of which is unable to activate its haemagglutinin, it is possible to select a mutant with a haemagglutinin which has become cleavable in these cells, even though the cleavage site itself has not been changed. The only mutation that was found was the replacement of a histidine by an arginine at a position approximately 10 Å distant from the cleavage site (*Rott* et al., 1984). The three dimensional structure of the haemagglutinin suggests that, because of this amino acid substitution, the stability of the cleavage site was changed. As a result, the cleavage site became exposed to the activating protease of the new host cell. These experiments are of general significance in that they show that influenza viruses with a broadened cell spectrum can be selected by adaptation to a novel host.

There is evidence that the acquisition of pathogenicity by an H5N2 virus responsible for a fowl pest-like outbreak in the USA in 1983 was caused by a similar event (*Kawaoka* et al., 1984). Comparative studies of different isolates indicate that during the epidemic the virus changed from being one of low pathogenicity to one of high pathogenicity. The haemagglutinin of the original low pathogenic virus had a limited cleavability, whereas the haemagglutinin of the highly pathogenic variant had become cleavable in a wide range of different host cells. Sequence analyses showed that the pathogenic variant possessed a cleavage site with the structure lysine–lysine–lysine–arginine, i.e. a cleavage site recognizable by ubiquitous cellular proteases. Surprisingly, the original low pathogenic virus had the same cleavage site, but this was apparently masked by a neighbouring oligosaccharide side-chain which was lost as the result of a point mutation.
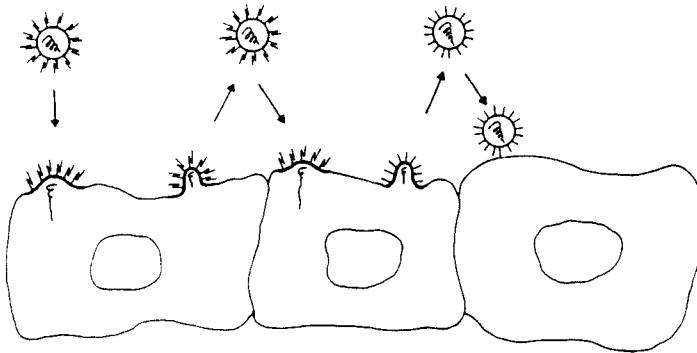
Fig. 2. Significance of proteolytic cleavage of the haemagglutinin for the spread of influenza viruses. Spread of the virus is prevented if the infectious virus, with cleaved haemagglutinin (ʎ), reaches cells which have proteases unable to mediate proteolytic activation. Virus particles with uncleaved haemagglutinin (|) are non-infectious.

Mechanism of pathogenicity for influenza viruses

This data, taken together, underlines the important role of proteolytic activation of the haemagglutinin in pathogenicity. Spread of an influenza virus, which is activated by proteolytic cleavage of the haemagglutinin in only a limited number of cells is inhibited as soon as the virus infects a cell which is unable to activate the HA (Fig. 2). The result is a local infection, which is seen regularly in mammals and also in birds infected by apathogenic influenza viruses. On the other hand, cleavability of the haemagglutinin in a wide spectrum of different cells, as is the case with the pathogenic avian influenza viruses permits a rapid production of infectious virus in all tissues to high amounts. This allows spread of the virus throughout the organism and results in a fatal systemic infection (*Rott*, 1979). The concept for this relatively simple mechanism of pathogenicity could be verified by *in vitro* experiments with organ cultures (*Bosch* et al., 1979; *Rott* et al., 1980).

Synergism between influenza viruses and bacteria in pathogenesis

More recently it has been found that some strains of *Staphylococcus aureus* secrete serine proteases that activate infectivity of influenza viruses by proteolytic cleavage of the haemagglutinin. The presence of the bacterial enzymes in the virus producing system enables the virus to undergo multiple step replication in cells which do not possess appropriate proteases. Correspondingly, co-infection of mice with influenza virus and *Staph. aureus* resulted in an enormous increase in titre in the lung with extensive lesions in the lung tissue and resulted in death of the mice (*Tashiro* et al., 1987a, b). Development of influenza pneumonia could be prevented by treatment of infected mice with a protease inhibitor (*Tashiro* et al., 1987c). These findings confirm again the significance of the haemagglutinin as a determinant of pathogenicity. They could also explain the high morbidity and mortality observed, e.g. in humans, after co-

infection with *Staph. aureus* and influenza viruses (*Robertson* et al., 1958). Finally, these observations also suggest that particularly serious infections in humans, as were observed during the "Spanish influenza" epidemic of 1918/19, are caused by a similar synergism between an influenza virus strain and a relatively harmless ubiquitous microorganism.

Significance of proteolytic cleavage of glycoproteins of other viruses

The fusogenic glycoproteins of many other viruses are synthesized as precursor molecules and are activated by proteolytic cleavage at arginine residues. It may be assumed that in these cases, as with influenza viruses, a hydrophobic region, which could act as a fusogen becomes exposed. Such glycoproteins include the F-protein of paramyxoviruses, the S-protein of coronaviruses and the envelope glycoprotein of retroviruses (Fig. 3). In agreement with the observation made with the haemagglutinin

Fusion proteins of paramyxoviruses

Sendai virus (1)	...Ala-Gly-Val-Pro-Gln-Ser- **Arg** ▽ -Phe-Phe...
NDV Italien (2)	...Gly-Gly-Arg-Arg-Gln-**Arg**- **Arg** ▼ -Phe-Ile...
NDV Ulster (2)	...Gly-Gly-Gly-Lys-Gln-Gly- **Arg** ▽ -Leu-Ile...
Simian virus 5 (3)	...Pro-Thr-**Arg**-**Arg**-**Arg**- **Arg** ▼ -Phe-Ala...
RS virus (4)	...Ser-**Lys**-**Lys**-**Arg**-**Lys**-**Arg**- **Arg** ▼ -Phe-Leu...

E2 protein of alphaviruses

SFV (5)	...Asn-Gly-Thr-Arg-His-**Arg**- **Arg** ▼ -Ser-Val...
Sindbis virus (6)	...Ser-Ser-Gly-Arg-Ser-**Lys**- **Arg** ▼ -Ser-Val...

S protein of coronaviruses

IBV (7)	...Gly-Thr-Arg-Arg-Phe-**Arg**- **Arg** ▼ -Ser-Ile...

Env protein of retroviruses

RSV (8)	...Thr-Gly-Ile-**Arg**-**Arg**-**Lys**- **Arg** ▼ -Ser-Val...
HIV1 (9)	...Pro-Thr-Lys-Ala-Lys-**Arg**- **Arg** ▼ -Val-Val...

Fig. 3. Examples of proteolytic cleavage sites in viral glycoproteins. The cleavage sites for proteases, which recognize only a single arginine, are labelled by (▽), and those for proteases with arginine-arginine or lysine-arginine specificity are symbolized by (▼). The amino acid sequences are taken from the following publications: (1) *Blumberg* et al., 1985; (2) *Nagai*, personal communication (NDV = Newcastle disease virus; the strain Italien is pathogenic for chicken, the strain Ulster is apathogenic); (3) *Paterson* et al., 1984; (4) *Collins* et al., 1984 (RS virus = respiratory syncytial virus); (5) *Garoff* et al., 1980 (SFV = Semliki Forest virus); (6) *Rice* and *Strauss*, 1981; (7) *Binns* et al., 1985 (IBV = infectious bronchitis virus); (8) *Schwarz* et al., 1983 (RSV = Rous sarcoma virus); (9) *Ratner* et al., 1985.

of influenza viruses, virus particles containing uncleaved precursor glycoproteins, can be obtained from appropriate cells if the cleavage site itself consists of a single arginine. An example is the F-protein of Sendai virus, and it should be pointed out that this was the first viral glycoprotein shown to be activated by proteolytic cleavage (*Homa* and *Ohuchi*, 1973; *Scheid* and *Choppin*, 1974). In this F-protein and those of other paramyxoviruses after cleavage a hydrophobic fusion peptide becomes exposed which has an amino acid sequence similar to the amino terminus of HA_2 of influenza viruses (*Gething* et al., 1978). Newcastle disease virus, another paramyxovirus, comprises a whole series of strains which, like the avian influenza viruses, differ widely in pathogenicity for chickens. Here again differences in pathogenicity can be correlated with the cleavability of the F-protein (*Nagai* et al., 1976). Sequence analyses have revealed that the apathogenic strains contain single arginine residues and the pathogenic strains paired basic residues at their cleavage site, exactly as has been observed with the avian influenza viruses (Fig. 3).

It is of interest that the infectivity of rotaviruses, which do not contain an envelope, is activated by trypsin-cleavage of one of its capsid proteins, VP3 (*Estes* et al., 1981). In genetic studies, VP3 was identified as a virulence factor (*Offit* et al., 1986). Since rotavirus strains differ from each other again by single arginine residues or paired basic amino acids at the cleavage site of VP3 (*López* et al., 1986), it is reasonable to assume that cleavability of this surface protein determines pathogenicity, although such a correlation has not yet been demonstrated. If bacteria present in the intestinal tract could induce a synergistic effect, as with influenza viruses, it would be difficult to detect such a correlation.

All in all, evidence is increasing that proteolytic activation of functionally important proteins may be a rather common determinant of pathogenicity.

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