Distribution and substrate specificity of intracellular proteolytic processing enzyme(s) for paramyxovirus fusion glycoproteins

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Intracellular proteolytic processing of fusion glycoprotein precursors (F₀) of paramyxoviruses, i.e. a virulent strain of Newcastle disease virus (NDV), parainfluenza virus type 3 (PIV3) and simian virus 5 (SV5), was examined in NALM6 and BSC40 cells and compared with that in LLCMK₂ cells to investigate the distribution of the virus-activating protease(s) among the cells and its substrate specificity. BSC40 cells lack a processing endoprotease of the neuropeptide precursor, pro-opiomelanocortin (POMC), which possesses multiple cleavage sites at pairs of basic residues, Lys-Arg and Arg-Arg, a motif similar to that found in the cleavage site of the F₀ proteins. In NALM6 cells, only small amounts of the F₀ protein of virulent NDV was cleaved whereas those of PIV3 and SV5 were efficiently cleaved. In BSC40 cells the F₀ proteins of these three viruses were cleaved normally as well as in LLCMK₂ cells. The processing inhibitors monensin, chloroquine and A23187 suppressed the F₀ cleavage in the three cell types. These results indicate that both NALM6 and BSC40 cells possess virus-activating proteases similar to that of LLCMK₂ cells, but suggest that the enzyme of NALM6 may be slightly different in its substrate specificity from those of BSC40 and LLCMK₂. The results also suggest that the virus-activating proteases are different in their distribution and substrate specificity from the processing enzyme of POMC.

Introduction

Paramyxoviruses have haemagglutinin–neuraminidase (HN) and fusion (F) glycoproteins in the envelope, which bind receptors on the cell surface and mediate membrane fusion between the viral envelope and host plasma membrane, thereby enabling entry of the viral genome into target cells (Morrison, 1988; Nagai et al., 1989). The F proteins of many paramyxoviruses, which are synthesized as an inactive precursor (F₀), are cleaved by a host endoprotease (or endoproteases) present in the trans cisternae of the Golgi apparatus (Morrison et al., 1985; Yoshida et al., 1986) into disulphide-linked F₁ and F₂ during intracellular transport. This proteolytic cleavage is essential for the fusion capacity of the F protein and hence for virus infectivity (Homma & Ohuchi, 1973; Nagai et al., 1976). The amino acid sequences of the F₀ proteins of Newcastle disease virus (NDV) (Toyoda et al., 1987) and simian virus 5 (SV5) (Paterson et al., 1984) predicted by nucleotide sequencing, and those of the N termini of their F₁ subunits revealed by direct amino acid sequencing (Richardson et al., 1980) indicate that the cleavage occurs at the carboxyl side of a pair or cluster of basic residues in the F₀ proteins. The proteolytic processing appears to be catalysed by a mechanism which is widespread in nature and utilized for the biosynthesis of bioactive cellular proteins such as hormones, neuropeptides, growth factors, receptors and several plasma proteins, as their precursors also have two or more basic residues at the cleavage site and the cleavage takes place in a similar location within the cells (Barr, 1991). However, there is no evidence at present concerning the identity of these processing endoproteases.

Sakaguchi et al. (1991) have recently identified a strong candidate for a virus-activating protease in the trans Golgi membranes of rat liver cells, which specifically cleaves in vitro the F₀ protein of a virulent strain of NDV with a putative cleavage site Arg-Arg-Gln-Arg-Arg (Toyoda et al., 1987). The endoprotease is membrane-bound, Ca²⁺-dependent and active at a broad pH range from 6 to 8. The inhibitor spectrum of the protease, together with the properties described above, indicates that the enzyme is very similar to KEX2, a prohormone-processing endoprotease of yeast (Julius et al., 1984; Fuller et al., 1988).
Paramyxoviruses with a pair or cluster of basic residues at the cleavage site in the F₀ protein, such as virulent NDV, measles virus, parainfluenza virus type 3 (PIV3) and SV5 can replicate in multiple cycles in a variety of cells and cause a systemic infection in the host or have the potential to cause this type of infection (Nagai et al., 1989). In addition, it has been shown by Tashiro et al. (1988) that a variant of Sendai virus (F1-R), whose F₀ protein possesses a putative cleavage site, Pro-Lys, can also undergo multiple cycles of replication in several cell lines and cause a generalized infection in mice. The virus-activating proteases for these viruses are therefore considered to be ubiquitous in cells of various tissues and organs.

We have found recently, however, that F₀ proteins of virulent NDV are cleaved to only a small extent in the lymphoid cell line NALM6 (Sakaguchi et al., 1991), suggesting that this cell lacks a virus-activating protease. In this communication the proteolytic processing of F proteins of virulent NDV, PIV3 and SV5, all containing F₀ cleavage sites with multiple basic residues, were examined in NALM6 and BSC40 cells and compared with that in LLCMK₂ cells to study the distribution of the virus-activating protease(s) among the cells and its substrate specificity. The F₀ protein of virulent NDV was hardly cleaved in NALM6 cells, whereas those of PIV3 and SV5 were efficiently cleaved in the cells. In BSC40 cells, which lack the processing endoprotease of the neuropeptide precursor pro-opiomelanocortine (POMC) with pairs of basic residues at the cleavage sites (Thomas et al., 1988), the F₀ proteins of these three viruses were cleaved normally as well as in LLCMK₂ cells. These results are discussed from the viewpoints of substrate specificity and distribution of virus-activating protease(s).

Methods

Cells and viruses. The human lymphoid cell line, NALM6, was grown in RPMI-1640 supplemented with 10% foetal calf serum (FCS), LLCMK₂ and MDBK cells, and BSC40 cells, a line of kidney epithelial cells from African green monkey, were grown in Eagle’s MEM containing 10% FCS. The cell line BSC40 was provided through the courtesy of Dr Gray Thomas, Oregon Health Sciences University, U.S.A. (Thomas et al., 1988). A virulent strain of NDV, Miyadera, was prepared in embryonated eggs. The C-243 strain of human PIV3 (Cook et al., 1959) and the W3 strain of SV5 (Choppin, 1964) were grown in LLCMK₂ and MDBK cells, respectively. The infectivities of these viruses were assayed by plaque titration on monolayers of LLCMK₂ cells (Nagai et al., 1972).

Virus infections of cells and labelling of infected cells with [³⁵S]methionine. Suspension cultures of NALM6 cells were infected with NDV or PIV3 at an input multiplicity of approximately 10 p.f.u. per cell or with SV5 at about 2 p.f.u. per cell. After an adsorption period of 1 h at 36 °C, the cells were washed three times with RPMI-1640 by low-speed centrifugation, and then incubated in RPMI-1640 at 36 °C. Confluent monolayers of LLCMK₂ or BSC40 cells were infected with NDV, PIV3 or SV5 at the same input multiplicities as used for NALM6 cells, and after 1 h adsorption the infected cells were incubated in MEM at 36 °C. For the labelling of infected cells with [³⁵S]methionine, the medium was removed at the indicated time after infection, and then MEM deficient in methionine and containing 10 µCi/ml of [³⁵S]methionine (1000 Ci/mmol, American Radiolabelled Chemicals) was added for appropriate periods. For chases, the radioactive medium was removed and the cells were incubated in MEM containing a 10-fold excess of methionine.

Partial purification of virions released from cells infected and labelled with [³⁵S]methionine. NALM6 and LLCMK₂ cells infected with NDV, PIV3 or SV5 were incubated at 36 °C in RPMI-1640 and MEM, respectively. At 6 h post-infection (p.i.), the medium was replaced with MEM in which the methionine concentration was reduced to one-tenth, containing 10 µCi/ml of [³⁵S]methionine. Chloroquine or A23187 was present in the medium during the labelling period when indicated. The medium of NDV-infected cells was harvested 26 h p.i. and that of PIV3- or SV5-infected cells was harvested 50 h p.i. The medium was centrifuged at 2000 r.p.m. for 5 min and then at 7000 r.p.m. for 20 min to remove remaining cells. Virions in the supernatant were pelleted through 1.5 ml of 25% (w/w) sucrose by centrifugation at 50 000g for 120 min at 4 °C in a Beckman SW55Ti rotor.

Immunoprecipitation and SDS-PAGE. Solubilization and immunoprecipitation of infected cells and virions labelled with [³⁵S]methionine were carried out as described by Sakaguchi et al. (1991) using rabbit antiserum against NDV or PIV3, or monoclonal antibodies (MAbs) against NDV, PIV3 or SV5 F protein and fixed Staphylococcus aureus. For the immunoprecipitation of the SV5 F protein, the lysis buffer described by Randall et al. (1987) was used, which consisted of 10 mM-Tris-HCl pH 7.4, 5 mM-EDTA, 0.5% NP40, 0.1% SDS, 0.65 M-NaCl and 1 mM-PMSF. MAb against the F proteins of NDV, PIV3 and SV5 were provided through the courtesy of Dr H. Kida, Hokkaido University, Japan (Abenes et al., 1986), Dr K. L. Van Wyke Coelingh, National Institutes of Health, U.S.A. (Van Wyke Coelingh et al., 1985) and Dr R. E. Randall, University of St Andrews, U.K. (Randall et al., 1987), respectively. The viral polypeptides were analysed by 10% SDS-PAGE and fluorography (Yoshida et al., 1982). The radioactivities of the F₀, F₁ and F₂ bands on the gel were quantified by densitometry of an X-ray film using the Argus-100 system (Hamamatsu Photonics) and the percentage of F₀ in the F proteins (F₀ + F₁ + F₂) was calculated. When F₁ was not resolved in the gel, the percentage was calculated from F₀/F₀ + F₂.

Inhibitors. Monensin and A23187 were purchased from Sigma and Calbiochem, respectively.

Results

Proteolytic processing of F proteins of virulent NDV, PIV3 and SV5 in NALM6 cells

Fig. 1 shows proteolytic processing of the F protein of virulent NDV in NALM6 cells and F proteins in virions produced from the cells. Most F proteins synthesized in NALM6 cells remained uncleaved (>66%), up to 180 min of the chase period and 72% of F proteins in the virions was F₀, indicating that the F protein of virulent NDV is cleaved in small amounts only in NALM6 cells.
Processing enzyme(s) of F proteins

(a) NALM6 cells (5 x 10⁶ cells) were infected with a virulent strain of NDV, Miyadera, and incubated in RPMI-1640 as described in Methods. The infected cells were labelled 7 h p.i. with 10 μCi/ml of [³⁵S]methionine for 15 min (lanes 1 and 3) and then chased for 15 min (lane 4), 30 min (lane 5), 60 min (lanes 2 and 6), 120 min (lane 7) or 180 min (lane 8) as described in Methods. The cells were processed for immunoprecipitation using anti-NDV (lanes 1 and 2) or anti-NDV F MAb (lanes 3 to 8) followed by PAGE and fluorography. (b) Virions released from NDV-infected and [³⁵S]methionine-labelled NALM6 cells (5 x 10⁷ cells) were partially purified as described in Methods. The viral polypeptides were analysed directly by PAGE (lane 1) or after immunoprecipitation using anti-NDV F MAb (lane 2). The F₂ polypeptide of NDV comigrated with the bromophenol blue dye and was not resolved under the condition used in these and the following experiments. The percentages of F₀/F₀ + F₁ were determined as described in Methods.

This extends the data reported recently by Sakaguchi et al. (1991) and indicates that NALM6 is a unique cell line in proteolytic processing of the viral F glycoprotein, since all the cultured cells examined so far, except for NALM6, efficiently cleave the F₀ protein of virulent NDV. Fig. 1 showed, however, that a small amount of cleaved F proteins were also present both in the cell and in the virion, and this was the case in repeated experiments.

To clarify whether processing proteases for F glycoproteins of the other paramyxoviruses are present in NALM6, we further examined proteolytic processing of the F proteins of PIV3 and SV5 in the cells by pulse-chase experiments. As shown in Fig. 2(a and b), most F₀ proteins of those viruses synthesized were cleaved in NALM6 cells within 120 min and the viruses produced from the cells were infectious. These results indicate that a virus-activating protease is present in NALM6 at least for PIV3 and SV5.

Effects of monensin, chloroquine and A23187 on the cleavage of F proteins of virulent NDV, PIV3 and SV5 in NALM6 cells

To obtain further information about the virus-activating protease of NALM6 cells, the effects of the processing inhibitors monensin, chloroquine and A23187 on the cleavage of the F proteins of virulent NDV, PIV3 and SV5 were examined.

(i) Effect of monensin

The sodium ionophore monensin, which is known to block intracellular transport of glycoproteins in the Golgi apparatus (Tartakoff, 1983), was used to localize the virus-activating protease in NALM6. NALM6 cells infected with virulent NDV, PIV3 or SV5 were labelled for 120 min with [³⁵S]methionine in the absence or presence of monensin, and proteolytic processing of the
(a) (b)

Fig. 2. Proteolytic processing of the F proteins of PIV3 (a) and SV5 (b) in NALM6 cells. NALM6 cells (5 × 10⁶ cells) were infected with PIV3 or SV5 as described in Methods. The infected cells were labelled 15 h (PIV3) or 12 h (SV5) p.i. with 10 μCi/ml of [35S]methionine for 30 min (a, lanes 1 and 3; b, lane 1) and then chased for 30 min (b, lane 2), 60 min (b, lane 3), 90 min (b, lane 4) or 120 min (a, lanes 2 and 4; b, lane 5) as described in the legend of Fig. 1. The viral polypeptides in the cells were immunoprecipitated by using anti-PIV3 (a, lanes 1 and 2), anti-PIV3 F MAb (a, lanes 3 and 4) or anti-SV5 F MAb (b, lanes 1 to 5) and analysed by PAGE and fluorography. The F₂ of PIV3 was not resolved under these conditions. The time used for PAGE of SV5 was shorter than that in subsequent experiments.

(ii) Effect of chloroquine

We have recently shown that the F protein cleavage of virulent NDV is partially inhibited in BHK cells treated with the weak bases chloroquine and ammonium chloride, and that significant amounts of F₀ proteins are incorporated into the virions released from the cells (Yoshida et al., 1989). These results suggest that an acidic environment in the trans and post-Golgi compartments of the cells (Anderson & Pathak, 1985) is required for the proteolytic processing of the F protein. The effect of chloroquine on the F protein cleavage of virulent NDV, PIV3 and SV5 in NALM6 and LLCMK₂ cells was therefore examined to compare the requirement for the intracellular low pH environment. NALM6 cells were infected with virulent NDV, PIV3 or SV5 and incubated with or without chloroquine. The viral polypeptides synthesized in the cells and those of virions released from the cells were analysed by PAGE. As shown in Fig. 4(a to c), the F protein cleavage of each virus in NALM6 cells was strongly inhibited in the presence of chloroquine and significant amounts of the F₀ proteins of PIV3 were incorporated into the virions. The SV5 particles released from the cells, however, could not be analysed owing to poor production from the cells even in the absence of chloroquine. In LLCMK₂ cells the F protein cleavage of NDV, PIV3 and SV5 was also inhibited in the presence of chloroquine and significant amounts of the F₀ proteins of these viruses were incorporated into virions released from drug-treated cells (Fig. 4d to f). These results suggest that an acidic environment in the trans and post-Golgi compartments is required for F protein cleavage of the three viruses both in NALM6 cells and in LLCMK₂ cells.
Processing enzyme(s) of F proteins

Since the protease(s) for intracellular processing of viral fusion glycoproteins containing a cleavage site consisting of multiple basic residues has been suggested to be Ca\(^{2+}\)-dependent (Klenk et al., 1984; Sakaguchi et al., 1991), the effect of the calcium ionophore A23187 on F protein cleavage was examined. NALM6 cells infected with virulent NDV, PIV3 or SV5 were incubated in medium with or without CaCl\(_2\) in the presence or absence of A23187. The infected cells were labelled with \([^{35}S]\)methionine for 120 min and analysed by PAGE. As shown in Fig. 5, the F protein cleavage of those viruses appeared to be slightly reduced in cells incubated in the medium without CaCl\(_2\) and was strongly inhibited in the presence of A23187. The PIV3 particles released from the drug-treated cells contained significant amounts of F\(_0\) proteins as compared with those from the untreated cells, but the particles of SV5 released from the drug-treated cells were too few to be analysed. We also obtained essentially the same results in LLCMK\(_2\) cells (data not shown). These results suggest that the proteolytic cleavage of F proteins both in NALM6 and in LLCMK\(_2\) cells is a Ca\(^{2+}\)-dependent process at least with virulent NDV and PIV3.

These results described above indicate that the virus-activating protease of NALM6 cells has properties indistinguishable from that of LLCMK\(_2\) cells according to experiments using monensin, chloroquine and A23187.

**Proteolytic processing of the F proteins of virulent NDV, PIV3 and SV5 in BSC40 cells**

It has been shown that BSC40 cells cannot process the murine POMC when its synthesis is directed by a vaccinia virus vector (Thomas et al., 1988). When the cells are co-infected with vaccinia viruses carrying the POMC gene and the yeast KEX2 gene, POMC is cleaved intracellularly at pairs of basic residues into a set of product peptides normally found in vivo, including mature \(\gamma\)-lipotropin and \(\beta\)-endorphin. These results suggest that the inability of BSC40 to process a prohormone precursor is due to the absence of a suitable endogenous processing protease. Thus, we examined the proteolytic processing of viral F proteins in BSC40 cells to investigate identity between the processing enzymes for viral F proteins and for POMC. Fig. 6 shows cleavage of the F proteins of virulent NDV, PIV3 and SV5 in BSC40 cells. All the F protein precursors synthesized were cleaved normally in the cells, and the cleavage of virulent NDV F protein in BSC40 cells was inhibited by monensin, chloroquine or A23187 (data not shown), indicating that BSC40 cells possess the paramyxovirus-activating protease indistinguishable from that of LLCMK\(_2\) cells. These results suggest that the processing protease(s) for viral F proteins which have a cleavage site of multiple basic residues is not identical with that for POMC.
Fig. 5. Effect of A23187 on cleavage of the F proteins of NDV (a), PIV3 (b) and SV5 (c) in NALM6 cells and F proteins in PIV3 particles released from the cells (b). NALM6 cells (5 × 10⁶ cells) were infected with virulent NDV, PIV3 or SV5 and incubated in MEM with (+) or without (−) CaCl₂ in the absence or presence of 0.1 μM-A23187 as indicated at the bottom of the figure. The infected cells were labelled for 120 min with [³⁵S]methionine as described in the legend of Fig. 3. The same amounts of CaCl₂ and A23187 were present during the labelling period. PIV3 particles were prepared from the medium of infected NALM6 cells (5 × 10⁷ cells) as described in Methods, which were labelled with [³⁵S]methionine in the absence (b, lane 4) or presence (b, lane 5) of 0.1 μM-A23187. The labelled cells and virions were processed for immunoprecipitation using anti-F MAbs against the respective virus and analysed by PAGE. The percentages of Fo/Fo + F₁ (+ F₂) (%) 83 90 95 50 71 95 8 69 29 62 80.

Fig. 6. Proteolytic processing of the F proteins of NDV (a), PIV3 (b) and SV5 (c) in BSC40 cells. Monolayers of BSC40 cells on 3.5 cm dishes were infected with virulent NDV, PIV3 or SV5 and labelled 7 h (NDV), 15 h (PIV3) or 12 h (SV5) p.i. for 30 min (lane 1) with 10 μCi/ml of [³⁵S]methionine and then chased for 30 min (lane 2) and 60 min (lane 3), respectively, as described in Methods. The cells were processed for immunoprecipitation using anti-F MAbs against the respective virus followed by PAGE and fluorography.

Discussion

In the present study we showed that only a small proportion of the F₀ protein of virulent NDV with a cleavage site of Arg-Arg-Gln-Arg-Arg was cleaved into F₁ and F₂ in the lymphoid cell line NALM6, whereas those of PIV3 and SV5 with cleavage sites of Pro-Arg-Thr-Lys-Arg (Spriggs et al., 1986) and Arg-Arg-Arg-Arg-Arg (Paterson et al., 1984), respectively, were processed normally in the cells. The cleavages were inhibited in NALM6 cells treated with monensin, chloroquine or A23187, and similarly in LLCM₂ cells. These results indicate that NALM6 cells possess a virus-activating protease which cleaves the F₀ proteins of PIV3 and SV5 specifically, probably at a pair or cluster of basic residues, and suggest that both processing proteases in NALM6 and LLCM₂ are Ca²⁺-dependent and present in the Golgi apparatus, probably in the trans compartment. The cleavage of virulent NDV F₀ in NALM6 cells, which was always observed in small amounts, was also inhibited by the three drugs, suggesting that the NDV F₀ proteins are partially cleaved in NALM6 cells by the same enzyme used for cleavage of the PIV3 and SV5 F₀ proteins. Thus, it is likely that NALM6 cells have...
a virus-activating protease similar to but slightly different in substrate specificity from that of other cells such as LLCMK₂. Other possibilities can not be excluded, however, if those cells were assumed to have two or more species of virus-activating proteases, that the enzyme for NDV is expressed to only a small degree in NALM6 cells, or that the enzyme activity is suppressed by an inhibitor in the cells.

Furthermore, the present results showed that BSC40 cells processed the F₀ proteins of virulent NDV, PIV3 and SV5 normally and that the processing was inhibited by monensin, chloroquine and A23187. These results indicate that the BSC40 cell is not unusual in its capability to cleave the paramyxovirus F₀ proteins, and it is apparent that the cell possesses a virus-activating protease(s) that cleaves a pair or cluster of basic residues in the F₀ protein. The virus-activating protease(s) of the BSC40 cell appears incapable of cleaving the expressed POMC at the sites of basic residues pairs, since the POMC could meet the virus-activating enzyme(s) present in the constitutive secretory pathway of the cell during its transport for the following reason. The expressed POMC and F₀ proteins of virulent NDV were both successfully processed by the yeast KEK2 protease expressed by vaccinia virus vectors in BSC40 (Thomas et al., 1988) and NALM6 cells (N. M. Innocencio, unpublished data), respectively, both of which have only a constitutive pathway.

The failure of cleavage of POMC by the virus-activating protease(s) in BSC40 suggests that, in addition to a pair of basic residues at positions -1 and -2 in the F₀ protein, counting from the cleavage site to the N terminus, an arginine residue at position -4, which is not seen in POMC, may be essential for cleavage by the virus-activating protease(s). In fact, most viral fusion proteins examined so far, which are intracellularly cleaved in various cell types, possess an arginine or lysine at position -4 in addition to a pair of basic residues at positions -1 and -2 (Nagai, 1990). Furthermore, the view is supported by data, obtained by site-directed mutagenesis, that at least four arginine residues are required for cleavage activation of the SV5 F₀ protein by host proteases (Paterson et al., 1989).

In summary, the present study has demonstrated that all the cells examined possess a virus-activating protease(s) for paramyxovirus (intracellular processing type), and that some minor variations of substrate specificity may occur among the enzymes of various cell lines. In addition, the virus-activating protease(s) has been found to be different from the enzyme that processes POMC in its distribution among cells and substrate specificity.

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References


tides with amino acid sequences similar to those at the N-termini of the F or HA viral polypeptides. *Virology* **105**, 205–222.


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