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Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease

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Many viruses have membrane glycoproteins that are activated at cleavage sites containing multiple arginine and lysine residues by cellular proteases so far not identified. The proteases responsible for cleavage of the hemagglutinin of fowl plague virus, a prototype of these glycoproteins, has now been isolated from Madin-Darby bovine kidney cells. The enzyme has a mol. wt of 85 000, a pH optimum ranging from 6.5 to 7.5, is calcium dependent and recognizes the consensus sequence R-X-K/R-R at the cleavage site of the hemagglutinin. Using a specific antiserum it has been identified as furin, a subtilisin-like eukaryotic protease. The fowl plague virus hemagglutinin was also cleaved after coexpression with human furin from cDNA by vaccinia virus vectors. Peptidyl chloroalkylketones containing the R-X-K/R-R motif specifically bind to the catalytic site of furin and are therefore potent inhibitors of hemagglutinin cleavage and fusion activity.

Key words: fowl plaque virus/furin/hemagglutinin/multibasic cleavage site/subtilisin-like endoproteases

Introduction

Endoproteolytic cleavage at arginine residues is a common post-translational modification of membrane and secretory proteins on the exocytotic transport route. Such proteins include precursors of peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell surface receptors and adhesion molecules (for review see Barr, 1991).

The available evidence indicates that the cellular proteases involved in the processing of these proteins are also responsible for the cleavage of many viral membrane proteins. Detailed information on the biological significance of proteolytic cleavage in a virus system has been obtained from studies on the hemagglutinin (HA) of influenza virus (for review see Klenk and Rott, 1988). Cleavage of the HA, which is essential for the ability of the virus to enter cells, proved to be an important determinant for the spread of infection through the organism and for virus pathogenicity. The mammalian and the apathogenic avian influenza virus strains have HAs that are cleaved only in a restricted number of cell types. These viruses therefore cause local infection. On the other hand, the pathogenic avian strains have HAs activated in a broad range of different host cells and, thus, cause systemic infection. There are several structural properties of the HA that determine the differential cleavability, but the key factor is the amino acid sequence at the cleavage site (Kawaoka and Webster, 1988; Ohuchi *et al.*, 1989, 1991; Khatchikian *et al.*, 1989; Li *et al.*, 1990). Mammalian and apathogenic strains have a single arginine at this site, and plasmin (Lazarowitz and Choppin, 1975), a factor X-like protease from allantoic fluid of chicken eggs (Gotoh *et al.*, 1990; Ogasawara *et al.*, 1992) and bacterial proteases (Tashiro *et al.*, 1987) have been identified as enzymes activating this type of HA. The HAs of the pathogenic strains, on the other hand, have long been known to contain multiple lysine and arginine residues at their cleavage sites (Bosch *et al.*, 1981; Kawaoka and Webster, 1988), and recently it has been shown that the consensus sequence R-X-K/R-R is critical for cleavage activation (Vey *et al.*, 1992).

The ubiquitous proteases responsible for the activation of the HA of pathogenic strains were not well understood. However, it was known that they are calcium dependent and have a neutral pH optimum (Klenk *et al.*, 1984), and that they can be inhibited by specific peptidyl chloroalkylketones (Garten *et al.*, 1989). Such enzymes appear to be highly conserved, since the HA of fowl plague virus (FPV), the prototype of these pathogenic avian strains is activated not only in virtually all mammalian and avian cells analyzed but also in invertebrate cells (Kuroda *et al.*, 1986). It was therefore interesting to find that a protease resembling these enzymes in its catalytic and other biochemical properties exists in the yeast *Saccharomyces cerevisiae* (Fuller *et al.*, 1988). This Kex2 protease cleaves its natural substrates, pro-α-factor and pro-killer toxin, at R-R and K-R sites, but it can also process mammalian hormone and neuropeptide precursors. Furthermore, several Kex2 analogues, furin/PACE (Fuller *et al.*, 1989; Hatsuzawa *et al.*, 1990; Wise *et al.*, 1990), PC2 (Seidah *et al.*, 1990; Smeekens and Steiner, 1990) and PC1/PC3 (Seidah *et al.*, 1991; Smeekens *et al.*, 1991) have recently been discovered in human and mouse tissues. These enzymes together constitute a new family of eukaryotic subtilisin-like endoproteases (Barr, 1991). In the present study we show that human furin expressed from cDNA activates the FPV HA by proteolytic cleavage. Furthermore, the activating enzyme present in Madin-Darby bovine kidney cells, a cell line allowing FPV replication *in vivo*, has been identified as a furin-like protease. These findings indicate that the proteases responsible for the activation of viral glycoproteins at multibasic cleavage sites are subtilisin-like enzymes.

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Results

FPV HA is cleaved by human furin expressed under the direction of a vaccinia virus recombinant

For the reasons outlined above, furin appeared to be a likely candidate for an activating enzyme of the FPV HA. To prove this hypothesis, HA cDNA and human furin cDNA isolated from a hepatoma (HepG2) cDNA library (Bresnahan *et al.*, 1990; Wise *et al.*, 1990) were expressed in CV-1 cells alone or in combination, using vaccinia virus recombinants as vectors (Figure 1). Lanes 1 and 2 demonstrate that, when expressed alone or together with the HA, furin can be detected as a 90–96 kDa band after immunoprecipitation as has been described before (Wise *et al.*, 1990). When HA expressed alone was analyzed by pulse–chase labeling at 4–6 h after infection, it was detected mainly in the form of subunits HA_1 and HA_2, since the precursor was rapidly cleaved by the proteolytic activity endogenous to CV-1 cells. However, in agreement with a previous report on murine β-nerve growth factor (Bresnahan *et al.*, 1990), there was an accumulation of the precursor HA late after infection. Thus, when pulse–chase labeled at 20 h post-infection, HA expressed alone persisted in the uncleaved form (Figure 1, lane 3). It was therefore possible to analyze the effect of over-expressed furin on HA processing. The results of an experiment in which HA was labeled in the same way after coexpression with furin are shown in lane 4. The observation that the HA is now predominantly present in the leaved form demonstrates that furin of human origin is able to process this glycoprotein.

Identification of a furin-like protein as the activating enzyme of MDBK cells

It was therefore of interest to find out if the endogenous protease responsible for HA activation in MDBK cells infected with FPV was related to furin, with which it shared such characteristics as calcium dependence and neutral pH optimum as pointed out above. Although it was known from our previous study that lysates of MDBK cells contained a HA activating enzyme (Klenk *et al.*, 1984), we were unable to detect furin in these lysates by the Western blot technique employing an immune serum that was generated against the catalytic domain of human furin expressed as a fusion protein in *Escherichia coli* (Wise *et al.*, 1990). Since the failure to detect furin by this approach might have reflected too low concentrations in MDBK cells, an attempt was made to purify furin and to monitor it in the enriched enzyme preparation (Table I). Three independent assays were used for enzyme identification throughout the purification procedure. These were cleavage analysis of the FPV HA using virions containing the precursor HA as substrate (Klenk *et al.*, 1984), cleavage analysis using the fluorogenic peptide RQRR-AMC which mimics as substrate the cleavage site and immune analysis employing the described immune serum.

The first step in the isolation procedure involved fractionation of cytoplasmic extracts of MDBK cells on discontinuous sucrose density gradients. Cell fractions containing plasma membrane, lysosomes, Golgi apparatus and endoplasmic reticulum have been characterized by specific marker enzymes. HA cleavage activity cosedimented with the Golgi fraction which presumably also included the *trans*-Golgi network, suggesting that the activating enzyme is located in one of these organelles (data not shown). Since it was known that the activating protease is membrane bound (Klenk *et al.*, 1984), *n*-octylglucoside was used for the solubilization of the Golgi fraction and employed in all further purification steps. Another essential factor in the isolation procedure was the use of the protease inhibitors leupeptin, E-64, PMSF and pepstatin. As shown below

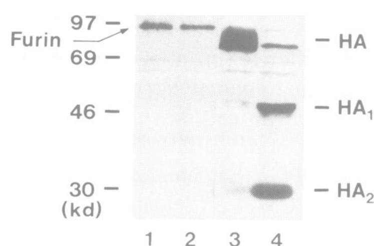

Fig. 1. Coexpression of human furin and HA by recombinant vaccinia viruses. CV-1 cells were labeled for 1 h with [^{35}S]methionine (100 μCi/ml) at 20 h p.i. with recombinant vaccinia viruses VVhFur and VV-HAwt, expressing the *fur* gene (Bresnahan *et al.*, 1990) and the HA gene of influenza virus A/FPV/Rostock/34 (H7N1) respectively. After 1 h chase with unlabeled methionine the cells were lysed, the vectorially expressed proteins immunoprecipitated with the respective antisera and analyzed by SDS–PAGE and fluorography. Lane 1, furin immunoprecipitated from cells infected with VVhFur and VV-HAwt; lane 2, furin immunoprecipitated from cells infected with VVhFur; lane 3, FPV HA immunoprecipitated from cells infected with VV-HAwt; lane 4, FPV HA immunoprecipitated from cells infected with VVhFur and VV-HAwt.

Table I. Purification of HA cleaving protease from MDBK cells

Purification step	Activity (units)	Total protein (mg)	Sp. act. (units/mg)
1. Microsomes	13 401	102.3	131
2. Sucrose gradient	12 015	53.4	225
3. DEAE–Sepharose CL6B	7830	8.7	900
4. Mono Q FPLC	1691	0.29	5.833
5. Superose 12 FPLC	600	0.057	10.526

1 unit: 1 ng HA cleaved per minute at 37°C.

Table II. Effect of inhibitors on HA cleaving protease of MDBK cells

Inhibitors	Concentration	Inhibition of HA cleavage (%)
DFP	1 mM	0
PMSF	1 mM	0
TLCK	1 mM	0
Leupeptin	1 mM	0
Aprotinin	1 mM	0
Iodoacetamide	1 mM	0
N-Ethylmaleimide	1 mM	0
Cadmium	1 mM	0
Cobalt	1 mM	0
E64	1 mM	0
Hydroxymercuribenzoic acid	1 mM	80
EDTA	50 mM	100
EGTA	50 mM	100
Phenanthroline	1 mM	0
Pepstatin A	50 μg/ml	0

Lysates of microsomes from MDBK cells were pre-incubated with the inhibitors in the presence of 10 mM $CaCl_2$. Aliquots of the pre-treated lysates were then incubated at 37°C for 30 min with [^{35}S]methionine-labeled FPV containing uncleaved HA. The incubation mixtures were dissolved in sample buffer and analyzed by SDS–PAGE. Cleavage of HA was densitometrically estimated from fluorographies.

(Table II), these inhibitors do not interfere with the HA activating enzyme, when analyzed in fresh cell lysates. They inactivate, however, other proteases that may degrade the HA activating protease or the virus used as substrate in the course of the isolation procedure. In the second step of this procedure, the solubilized Golgi proteins were separated by ultracentrifugation in a continuous sucrose density gradient, followed by anion exchange chromatography in step 3. In step 4, the material was subjected to Mono Q FPLC. Figure 2 demonstrates that the fractions containing the HA activating protease also cleave the fluorogenic tetrapeptide RQRR-AMC, whereas another proteolytic activity specific for RR-AMC has been separated. The fluorogenic peptide RVRR-AMC had the same specificity as RQRR-AMC, but was ~5-fold more effective (data not shown). In the final step involving gel filtration by Superose 12 FPLC further purification of the HA activating protease was obtained (Figure 3). These results therefore indicate that MDBK cells contain, in addition to the protease activating HA, other proteases specific for multiple basic residues that have been separated during the purification procedure. As has been pointed out above, the protease fractions obtained throughout the isolation procedure have been monitored for their immunoreactivity with an antiserum specific for human furin by Western blot analysis. Whereas furin-related proteins could not be detected by this approach in MDBK cell lysates or at the initial purification steps, a single protein band (mol. wt 85 000) was observed after Mono Q and Superose 12 FPLC that was copurified with HA cleaving activity (Figures 2 and 3). It has to be pointed out that this protein has a smaller mol. wt (85 000) than human furin expressed in CV-1 cells (96 000) (Figure 1). These results indicate that MDBK cells contain a furin-related protein and they strongly suggest that this protein activates HA.

Our previous findings had already indicated that correct cleavage of the FPV HA by crude MDBK lysates occurred only at neutral pH (Klenk *et al.*, 1984). We have therefore determined the pH optimum of the enzyme preparation obtained after Superose 12 chromatography using RQRR-AMC as substrate. As shown in Figure 4, highest activity was observed between pH 6.5 and 7.5. This finding supports the concept that the FPV HA is activated in MDBK cells at pH values close to neutrality.

Endopeptidases are divided into four classes, serine- (EC 3.421), cysteine- (EC 3.422), aspartate- (EC 3.423) and metalloproteinases (EC 3.424), which can be discriminated by their sensitivity to specific inhibitors. We have analyzed the effect of these inhibitors on cleavage of the HA by the MDBK cell protease (Table II). Cleavage was inhibited by EDTA and EGTA reflecting the calcium dependence of the enzyme (Klenk *et al.*, 1984). However, the third inhibitor of metalloproteinases, phenanthroline, was ineffective. Of the inhibitors specific for cysteine proteinases, iodo-

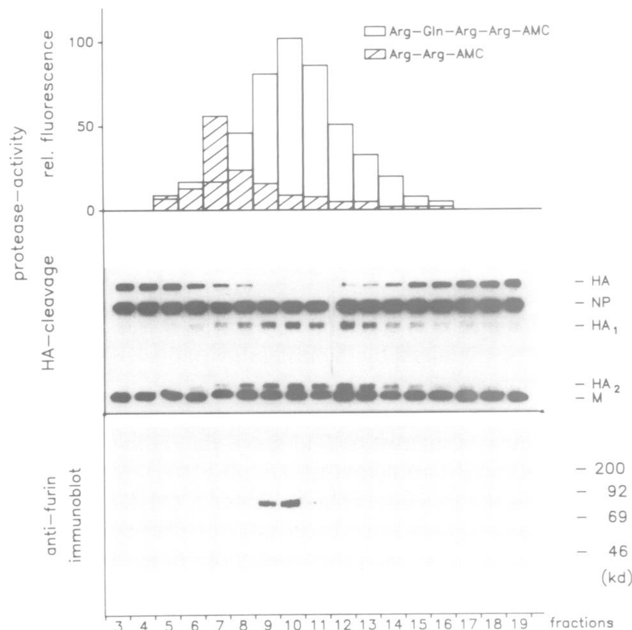

Fig. 2. Ion exchange chromatography of the HA activating protease of MDBK cells. Fractions containing protease activity obtained after DEAE–Sepharose CL6B chromatography were loaded on a Mono Q column. Proteins were separated by FPLC using a 0–300 mM NaCl gradient. **Upper panel**, protease activities of each fraction were tested by incubation with the fluorogenic peptidyl-7-amido-3-methylcoumarins indicated. **Middle panel**, protease activity was monitored by cleavage of HA after incubation of fractions from Mono Q with [^{35}S]methionine-labeled A23187-FPV. Viral proteins were analyzed by SDS–PAGE and fluorography. **Lower panel**, aliquots of Mono Q fractions were subjected to SDS–PAGE, transferred to a nitrocellulose membrane and analyzed by immunoreaction using antiserum against furin.

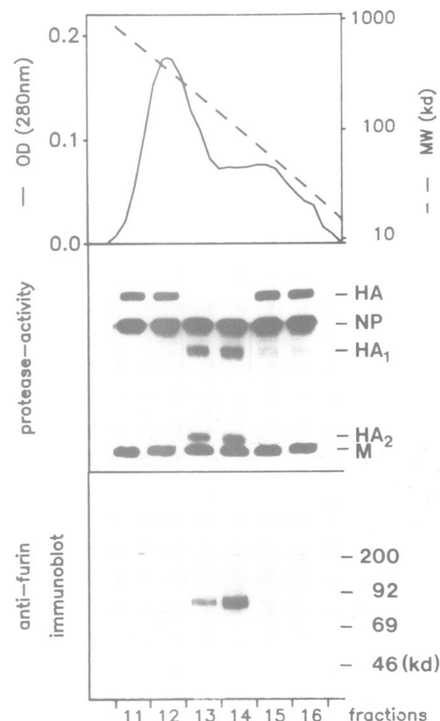

Fig. 3. Gel filtration of the HA activating protease MDBK cells. Protease fractions collected from the Mono Q column were separated by FPLC on a Superose 12 column. Eluted fractions (1 ml) of the chromatography containing HA cleaving activities are shown. **Upper panel**, protein profile of the eluate (solid line) and mol. wt standards (in kDa dextran blue 3000, ferritin 440, catalase 232, aldolase 158, albumin 67, ovalbumin 43, chymotrypsinogen 25, RNase 13.7; dashed line) were measured by optical density at 260 nm. **Middle panel**, protease activity was monitored by analyzing the cleavage of HA using [^{35}S]methionine-labeled A23187-FPV. **Lower panel**, aliquots of FPLC fractions were subjected to SDS–PAGE and immunoblotted with antiserum against furin.

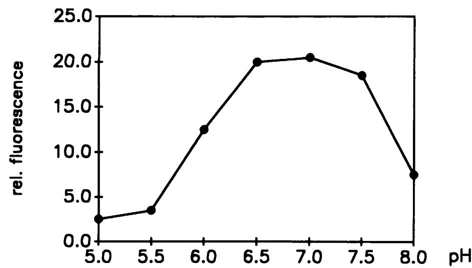

Fig. 4. pH optimum of the HA activating protease of MDBK cells. The activity of the protease obtained after FPLC on Superose 12 was determined in 100 mM sodium acetate buffer, ranging between pH 5.0 and 6.5, and 100 mM HEPES- and 100 mM Tris buffer ranging between pH 6.5 and 8.0. Boc-Arg-Gln-Arg-Arg-AMC was used as substrate. The relative fluorescence indicates the proteolytic activity.

acetamide, N-ethylmaleimide, cobalt chloride, cadmium acetate, E64 and hydroxymercuribenzoate, only the last one was effective. Neither pepstatin, an inhibitor for aspartate proteinases, nor the serine protease inhibitors DFP, PMSF, TLCK, TPCK, leupeptin and aprotinin interfered with cleavage. The ineffectiveness of PMSF was not expected since this compound has been reported to inhibit human furin (Bresnahan *et al.*, 1990). These results indicate that the MDBK cell protease, although a furin-like enzyme and thus belonging to the serine proteinases, does not have the inhibitor specificity typical for this class of endopeptidases.

Inhibition of cleavage activation by sequence-specific peptidyl chloromethylketones

We have previously shown that, unlike peptidyl chloromethylketones containing a single arginine, compounds with paired basic amino acids inhibit cleavage activation of the FPV HA and, thus prevent multiple cycles of virus replication (Garten *et al.*, 1989). Since it became clear that the consensus sequence has an additional arginine four amino acids upstream of the cleavage site (Vey *et al.*, 1992), peptidyl chloromethylketones with an arginine in this position have now also been synthesized and tested for their activity to inhibit cleavage by the MDBK cell protease. Most of these inhibitors were acylated to increase cellular uptake (Garten *et al.*, 1989). In the experiment shown in Figure 5, we analyzed the effect of such inhibitors using A23187 virions with uncleaved HA that had been incubated *in vitro* with the protease preparation obtained after Superose 12 chromatography. As shown before, the dibasic compound decFAKR-CMK inhibits at a 1 mM concentration. The new compound decREIR-CMK which has a single basic residue next to the cleavage site but an additional arginine at position −4 resembles in its inhibitory potential the other dibasic compound. However, when a pair of basic residues at positions −1 and −2 is combined with an arginine at position −4, as is the case with decREKR-CMK, there was an increase in sensitivity by a factor of ~1000.

We have also analyzed the effect of these inhibitors on *in vivo* cleavage of HA in FPV-infected MDBK cells (Figure 6). Again decREKR-CMK is the most efficient inhibitor. The data show also that distinct cleavage inhibition, as occurring at 100 μM decREKR-CMK, is not paralleled by a comparable reduction of hemagglutination titre in the medium. Thus, at appropriate concentrations, peptidyl chloromethylketones inhibit cleavage without interfering with particle formation. Two analogues of decFAKR-CMK were

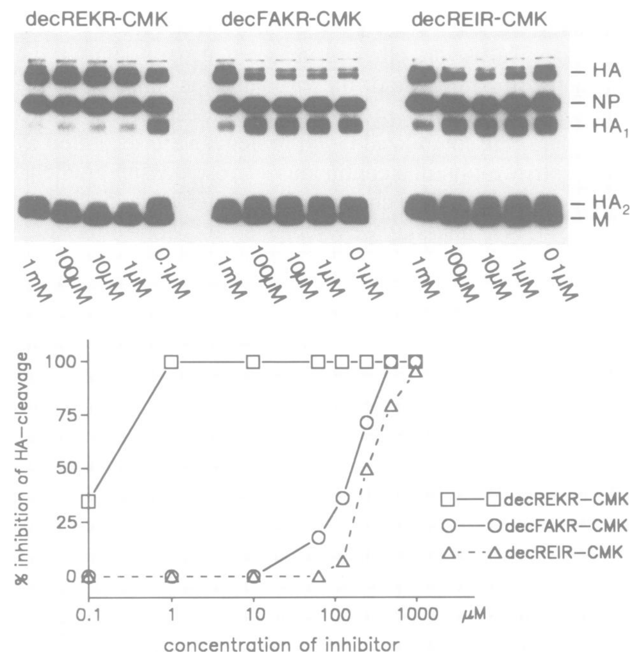

Fig. 5. Inhibition of the HA activating protease of MDBK cell by specific decanoylated peptidyl chloromethylketones. Purified HA activating protease was incubated with the inhibitors for 30 min at 37°C and analyzed for residual HA cleaving activity using A23187-FPV as substrate. Fluorographies of viral proteins (**upper panel**) and the densitometrical evaluation of HA cleavage as shown (**lower panel**).

also analyzed: decFAKR-S^+, a proteinase inhibitor (Shaw, 1988), in which the chloromethylketone group had been replaced by a sulfonium group, and palFAKR-CMK, in which the fatty acid had been changed from decanoic to palmitic acid. As shown in Figure 6, neither modification improved the inhibitory effect of decFAKR-CMK. It should be pointed out that ~100-fold higher concentrations of decREKR-CMK had to be used here than in experiments where the inhibitory effects on *in vitro* cleavage (Figure 5) and on fusion activity (see below, Figure 7) were analyzed. This may be explained by the instability of decREKR-CMK which requires relatively high concentrations in long-time experiments like this one.

It was then of interest to find out if the inhibitory effect of the peptidyl chloromethylketones also interfered with the biological activities of the HA. To this end, polykaryocytosis in BHK 21-F cells induced by HA at low pH was analyzed as depending on inhibitor concentration. Figure 7 demonstrates that complete inhibition of cell fusion was again obtained at micromolar concentrations of decREKR-CMK, whereas decFAKR-CMK and decRAIR-CMK had to be present at 100- to 1000-fold higher concentrations to produce the same effect. It is therefore clear that the peptidyl chloromethylketones with the correct amino acid sequence, while not interfering with hemagglutination and thus receptor-binding (Figure 6), specifically block the cleavage-dependent fusion activity of the HA.

Taken together these data indicate that decREKR-CMK is the most potent one of the inhibitors of HA cleavage analyzed. The amino acid sequence of this inhibitor is, in essence, identical to that of the fluorogenic compound RQRR-AMC which, as shown in Figure 2, is a specific

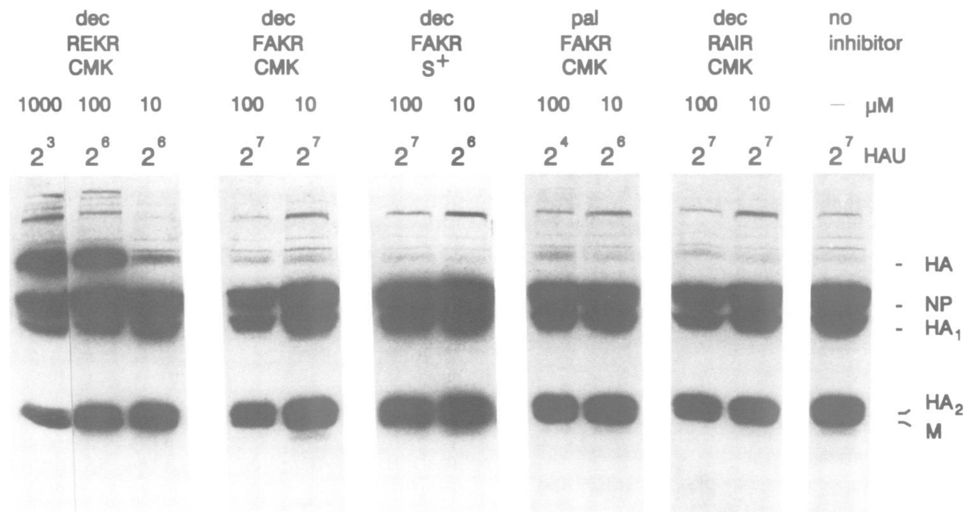

Fig. 6. Effect of various peptidyl chloroalkylketones on HA cleavage in cells infected with FPV. Inhibition of HA cleavage by the acylated peptidyl chloroalkylketones and the sulfonium compound was analyzed at 10, 100 and 1000 μM concentrations respectively, in chick embryo cells infected with FPV/Rostock/34 (H7N1) (10^9 p.f.u./ml). The cleavage patterns of the FPV HA were analyzed in infected cells by SDS–PAGE and fluorography. Hemagglutination titers of virus released into the medium are also indicated.

substrate for the furin-like protease from MDBK cells. These observations strongly suggest that decREKR-CMK interferes with cleavage activation of the FPV HA, because it specifically inhibits this protease. To strengthen further this concept, a radioiodinated peptidyl chloromethylketone of the structure [^{125}I]YARAKR-CMK was synthesized which could be used for affinity labeling studies. Figure 8 shows an experiment in which the fractions obtained from the Superose 12 column in the final purification step of the MDBK protease were analyzed with this probe. Two major bands (85 and 69 kDa) were radiolabeled (Figure 8, middle panel). The 85 kDa protein was identified as the furin-like protease by immunoprecipitation (Figure 8, lower panel). The 69 kDa band was present throughout the gradient and has also been found when affinity label not coupled to protein was analyzed. None of the minor bands seen comigrated with the protease activity. These observations indicate that, in the enzyme preparation obtained from MDBK cells, only the furin-like protein is able to recognize specifically the cleavage site of the HA. Thus, clear evidence has been obtained that this protein which has been identified now as bovine furin is responsible for HA activation in MDBK cells.

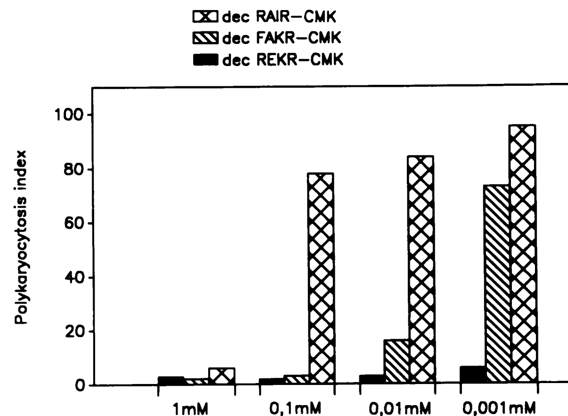

Fig. 7. Effect of peptidyl chloromethylketones on fusion of BHK21-F cells induced by FPV. Confluent monolayers of BHK21-F cells were infected with FPV/Dutch/27 (H7N7) (Dobson variant) and incubated for 6 h with the peptidyl chloromethylketones at the concentrations indicated. The cells were then kept for 2 min at pH 5.5 and analyzed for polykaryon formation as described in Materials and methods. The polykaryocytosis indices (PI) (percentage of total nuclei of a monolayer present in polykaryons) are indicated. PI of infected cells without inhibitor: $\geq$95. PI of uninfected cells: $<$5.

Discussion

We show here that the HA of FPV, a viral membrane glycoprotein with a multibasic cleavage site, is activated by furin. This conclusion was reached by two different approaches. Firstly, we could demonstrate that the FPV HA was cleaved, when it was coexpressed with human furin using vaccinia virus vectors. Expression from cDNA was also used to show that furin of human and murine origin is able to activate three secretory proteins: von Willebrand factor (Wise *et al.*, 1990), renin (Hosaka *et al.*, 1991), and β-nerve growth factor (Bresnahan *et al.*, 1990). Secondly, bovine furin was identified as the endogenous protease responsible for cleavage activation of HA in MDBK cells, a bovine kidney cell line allowing *in vivo* replication of FPV. Since furin is present in MDBK cells in amounts below the threshold of detection, the enzyme had to be enriched by conventional cell fractionation and protein purification procedures. Although purification to homogeneity has not been accomplished, the protease was sufficiently concentrated to allow unambiguous identification by a specific antiserum and by affinity labeling with a radioactive peptidyl chloromethylketone mimicking the cleavage site of the HA. We have, thus, not only shown that furin over-expressed from a foreign gene can activate at R-X-K/R-R cleavage sites, but we have demonstrated, to our knowledge for the first time, that the indigenous enzyme responsible for this type of proteolytic activation in a given cell is furin.

As a member of the family of eukaryotic subtilisin-like endoproteases, bovine furin shows many features typical for these enzymes. These include, above all, the substrate specificity for multibasic cleavage sites which has been analyzed here by using peptidyl coumarins as fluorogenic

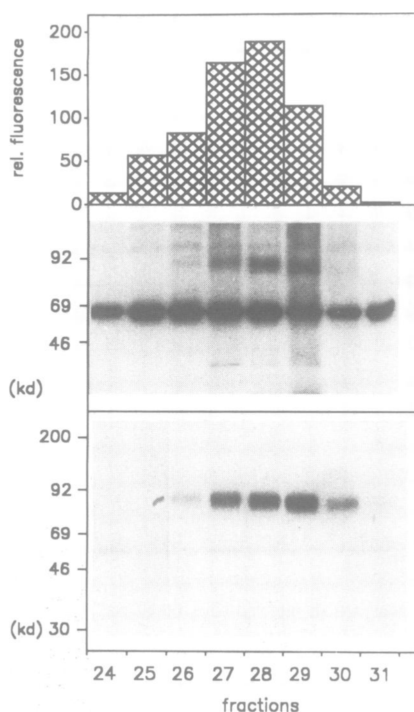

Fig. 8. Affinity labeling of the HA activating protease of MDBK cells with radioactive peptidyl chloromethylketone. Aliquots of 0.5 ml fractions eluted from the Sepharose 12 FPLC column were incubated at 37°C for 30 min with [^{125}I]YARAKR-CMK at a concentration of 0.05 μM and a sp. radioact. of 2000 Ci/mmol, immunoprecipitated with anti-furin serum, and subjected to SDS−PAGE. **Upper panel**, HA protease activity was monitored using Boc-Arg-Val-Arg-Arg-AMC as substrate. **Middle panel**, column fractions were analyzed by SDS−PAGE followed by autoradiography. **Lower panel**, fractions were immunoprecipitated with the anti-furin serum prior to SDS−PAGE.

substrates and by peptidyl chloroalkylketone inhibitors. The results indicate that bovine furin specifically recognizes the cleavage sites of the structure R-X-K/R-R, as has been observed before with murine furin (Hosaka *et al.*, 1991). These observations are also in agreement with the concept that influenza virus HA requires this consensus sequence for high cleavability (Vey *et al.*, 1992). Other features shared by bovine furin and the other subtilisin-like proteinases are calcium dependence, neutral pH optimum, sensitivity to hydroxymercuribenzoate and insensitivity to many inhibitors of other serine proteinases. On the other hand, bovine and human furin differ in their mol. wt. There are also differences in substrate specificity between the subtilisin-like proteases. Furin differs from Kex2, the prototype of these enzymes, by the requirement of arginine at position −4, in addition to the basic residues at positions −1 and −2 of the cleavage site (Barr, 1991), and studies with yeast extracts have shown that Kex2 is unable to cleave the FPV HA (data not shown). The differential substrate specificity also separates furin from PC2 and PC1/PC3 which again cleave only at dibasic cleavage sites (Hosaka *et al.*, 1991). PC2 differs also in its pH optimum at 5.5 (Shennan *et al.*, 1991).

Unlike PC2 and PC1/PC3 which are believed to be responsible for cleavage of pro-hormones and neuropeptides in secretory vesicles of the regulated secretory pathway, furin appears to be a component of the constitutive secretory pathway (Barr, 1991). It has long been known that cleavage of the FPV HA is a late post-translational process, but still occurs within the cell (Klenk *et al.*, 1974). More recently evidence has been obtained that the *trans*-Golgi network may be the cleavage compartment (De Curtis and Simons, 1989). Although the data presented here do not allow an exact allocation of the cleaving enzyme, they are compatible with the concept that the Golgi complex may be involved. Evidence has also been obtained that the glycoprotein of Rous sarcoma virus (Perez and Hunter, 1987), the prM protein of flaviviruses (Randolph *et al.*, 1990), the F proteins of mumps (Yamada *et al.*, 1988) and Newcastle disease virus (NDV) (Morrison *et al.*, 1985) are activated in the Golgi apparatus or the *trans*-Golgi network. Furthermore, a calcium-dependent protease capable of activating the F protein of NDV has been isolated from *trans*-Golgi membranes of liver cells, but its identity has not been established yet (Sakaguchi *et al.*, 1991). The neutral pH optimum of furin and the requirement of pH 7 for HA cleavage might argue against the Golgi apparatus or the *trans*-Golgi network as the cleavage compartment, since the intracisternal milieu of these organelles has been reported to be acidic (Anderson and Pathak, 1985; Griffith and Simons, 1986). However, firstly it should be pointed out that the pH optimum of furin is broad, ranging from pH 6.5 to 7.5. Furthermore, evidence has been obtained recently that at low pH the FPV HA undergoes conformational alterations interfering with correct cleavage and that, in infected cells, ion channels formed by the M2 protein of influenza virus raise the pH in the *trans*-Golgi network (Sugrue *et al.*, 1990). The virus may therefore be able to adjust the intracisternal milieu to optimal conditions for HA processing.

FPV is the prototype (Klenk and Rott, 1988) of a long list of other viruses that also contain glycoproteins with R-X-K/R-R cleavage sites. Among these viruses, which belong to many different taxonomic families, are important pathogenic agents, such as measles virus (Richardson *et al.*, 1986), mumps virus (Waxham *et al.*, 1987), canine distemper virus (Barrett *et al.*, 1987), respiratory syncytial virus (Collins *et al.*, 1984; Elango *et al.*, 1985), yellow fever virus (Rice *et al.*, 1985), HIV (Ratner *et al.*, 1985), human cytomegalovirus (Spaete *et al.*, 1990) and varicella-zoster virus (Keller *et al.*, 1986). With many of these viruses evidence has been obtained that glycoprotein cleavage is of biological significance, as is the case with influenza virus. It is therefore fair to assume that furin plays an important role in the life-cycle of these viruses and in their interplay with the host. On the other hand, we have presented evidence here that MDBK cells contain, in addition to furin, other proteases recognizing multibasic cleavage sites. Although these activities proved to be different from the protease cleaving the HA, it cannot be ruled out that they activate other viral glycoproteins. Furthermore, there is evidence for variations of furin between species, and they may also exist on the level of tissues and cells. Obviously, such variations may be important for organ tropism, host range and pathogenicity of any of these viruses. It also remains to be seen if infections with these viruses can be controlled by the peptidyl chloromethylketones. Since these inhibitors are also likely to interfere with the biosynthesis of many important cellular proteins, they are usually considered toxic. However, we have already shown previously that dibasic chloromethylketones, when interfering with FPV replication

in tissue culture, cause relatively little cell damage (Garten *et al.*, 1989). Since our new compounds containing the full consensus sequence have a significantly higher efficiency as judged by their capacity to inhibit cell fusion, it should be interesting to test their therapeutic potential.

Materials and methods

Cell cultures and viruses

BHK21-F, CV-1, MDBK and primary chick embryo cells were grown in Dulbecco's medium containing 10% fetal calf serum. The Rostock strain of fowl plague virus, influenza A/FPV/Rostock/34 (H7N1) and the Dobson variant of the Dutch strain, influenza A/FPV/Dutch/27 (H7N7) were used. Seed stocks were grown in eggs. To obtain radioactive virus with uncleaved HA for the protease assays, the Dutch strain was grown in BHK21-F cells in the presence of [^{35}S]methionine and 0.1 μM ionophore A23187 (Calbiochem, Frankfurt/M, Germany) added to reinforced Eagle's medium (REM) lacking calcium ions and depleted of methionine (Klenk *et al.*, 1984; Garten *et al.*, 1989). For inhibition assays the Rostock strain was propagated in chicken embryo cells.

Expression of recombinant vaccinia viruses in CV-1 cells

Confluent CV-1 cell monolayers were infected either with VV-HAwt (C.Roberts, H.-D.Klenk and W.Garten, manuscript in preparation), or with VV-HAwt and VVhFUR (Bresnahan *et al.*, 1990) or with VVhFUR, each at a m.o.i. of four. At 18 h after infection, the cells were starved of methionine in MEM without methionine for 60 min, each cell culture was labeled with 100 μCi [^{35}S]methionine for 60 min, and the radioactive label was chased by adding MEM containing 10 mM methionine for another 60 min. Then, the media were removed and the cells were washed twice with ice-cold phosphate buffered saline and lysed for immunoprecipitation.

Immunoreactions

For immunoblotting, samples were mixed with an equal volume of 2-fold concentrated sample buffer (500 mM dithiothreitol, 4% SDS, 10% glycerol, 62.5 mM Tris−HCl, pH 6.8, 0.2% bromophenol blue), boiled for 3 min, and subjected to electrophoresis on 12% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes by electrophoresis. The blot was incubated with rabbit anti-furin serum at 1:2000 dilution. The bound antibodies were detected using biotinylated anti-rabbit IgG and streptavidin-biotinylated horse-radish peroxidase complex (Amersham-Buchler, Braunschweig, Germany) and 4-chloro-1-naphthol as substrate (Kuroda *et al.*, 1986). For immunoprecipitation, 20 μl samples were incubated with either 2 μl rabbit anti-FPV serum or 2 μl rabbit anti-furin serum (diluted 1:10) and 25 μl of a suspension of protein A−Sepharose CL-4B (Sigma, Deisenhofen, Germany) in 500 μl RIPA buffer (1% Triton X-100, 0.1% SDS, 1% deoxycholate, 10 mM EDTA, 20 mM Tris−HCl, pH 7.6) at 4°C for 16 h. Subsequently, the immune complexes were washed three times with RIPA buffer and suspended in sample buffer for SDS−PAGE.

Protease assays

For fluorometric assays, synthetic peptide derivates mimicking basic cleavage sites have been used as substrates. Boc-RQRR-AMC (=*N*-α-*t*-butyloxy-carbonyl-L-arginyl-L-glutaminyl-L-arginyl-L-arginine-7-amido-4-methyl-coumarin) manufactured as customer synthesis and Z-RR-AMC (=*N*-α-benzyloxycarbonyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin) were purchased from Bachem Biochemica, Heidelberg, Germany. Boc-RVRR-AMC (=*N*-α-*t*-butyloxycarbonyl-L-arginyl-L-valyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin) was purchased from peptide Institute Inc., Tokyo, Japan. Each substrate was kept as 100 μM stock solution in 50 mM HEPES buffer, pH 7.3, containing 10 mM $CaCl_2$ and 1% *n*-octyglucoside. Fluorogenic substrates (200 μl of stock solutions) were incubated with protease samples in a total volume of 2010 μl for 2 h at 37°C. The liberated amido-4-methylcoumarin was fluorometrically measured at an excitation at 380 nm and an emission at 460 nm (Julius *et al.*, 1984). Protease activities were expressed by relative fluorescence based on 7-amido-4-methylcoumarin of the same concentration as the given substrate.

When uncleaved HA was used as substrate, protease samples (5 μl) were incubated with 5 μl [^{35}S]methionine-labeled virus at 37°C for 30 min. To protect viral proteins from non-specific degradation, 0.5 μl of lipid extract (see below) had to be added to incubation mixtures containing detergent. After SDS−PAGE under reducing conditions, the ratio of cleaved to uncleaved HA was determined on fluorographies using a Bio-Rad Model 620 Videodensitometer. Proteolytic activity was either measured as a percentage of final HA cleavage reached after 2 h incubation at 37°C, or defined by HA cleaving units. One unit is defined as 1 ng of cleaved HA obtained in 1 min at 37°C at a concentration of 0.2 μg uncleaved HA per μl.

Extraction of lipid from MDBK cells

Wet MDBK cells obtained from 10 dishes (14 cm in diameter) were stirred with 20 ml chloroform and 10 ml methanol at 0°C for 20 min and centrifuged at 1500 *g* for 30 min. The extracted lipids were dried under nitrogen and suspended in 100 μl 50 M HEPES, pH 7.3, 10 mM $CaCl_2$, 2% *n*-octylglucoside.

Preparation of furin samples from MDBK cells

Routinely, MDBK cells grown to confluency in 300 Petri dishes (14 cm diameter) were scraped from the plastic, washed with PBS three times, and collected by centrifugation at 500 *g*. Cells were suspended at 0°C in RSD medium containing 10 mM Tris−HCl, pH 7.4, and disrupted by 30 strokes in a Dounce homogenizer. Cell nuclei were removed by centrifugation at 2000 *g* for 20 min, and the microsomal fraction was pelleted in a SW28 rotor (Beckman) at 100 000 *g* for 30 min. Afterwards the pellet was resuspended in 4 ml 50 mM HEPES, pH 7.3, containing 10 mM $CaCl_2$ and laid on a discontinuous sucrose gradient (3 ml 65%, 7 ml 60%, 6 ml 45%, 6 ml 40% and 7 ml 25% w/w) in the same buffer. Subcellular fractions were separated by ultracentrifugation in a SW28 rotor at 25 000 r.p.m. for 16 h and identified by the following marker enzymes: 5′ nucleotidase and alkaline phosphatase (Engstrom, 1961) for plasma membranes, arylsulfatase (Chang *et al.*, 1981) and acid phosphatase (Engstrom, 1961) for lysosomes, galactosyltransferase (Fleischer *et al.*, 1969) for Golgi membranes, and glucose-6-phosphatase (Aronson and Touster, 1974) for endoplasmic reticulum. The Golgi fraction containing the HA cleaving activity was solubilized by adding 1-*o*-*n*-octyl-β-D-glucopyranoside (Boehringer Mannheim, Germany) at a detergent to protein ratio of 1.7:1 and stirring for 1 h at 4°C. After removal of insoluble material by centrifugation at 1000 *g*, the supernatant containing HA cleaving activity was loaded on a 5−25% (w/w) sucrose gradient containing 50 mM HEPES pH 7.3, 10 mM $CaCl_2$ and 1% *n*-octylglucoside, and centrifuged in a SW41 rotor (Beckman) at 35 000 r.p.m. for 16 h. The gradient was fractionated, 50 drops each fraction. Fractions containing HA cleaving activity were applied to a DEAE−Sepharose CL-6B column (Pharmacia, 4 cm long, 1.5 cm in diameter), which was equilibrated with the HEPES−calcium−octylglucoside buffer containing the protease inhibitors leupeptin (10 μM), *trans*-epoxysuccinyl-1-leucylamido (4-guanidino) butane (E64, Sigma) (10 μM), phenylmethylsulfonyl fluoride (PMSF) (0.4 mM) and pepstatin A (Sigma) (0.4 mM). The column was extensively washed with this buffer and then eluted with the same buffer containing 300 mM NaCl. The eluted protease containing fractions, were unified and concentrated by using a Centricon 30 microconcentrator (Amicon, Witten, Germany). The protease was excluded from the gel matrix. Two further steps of FPLC were used to isolate the HA cleaving enzyme from other proteolytic activities. First, HA cleaving fractions were applied to strong anionic exchange chromatography on a Mono Q HR5/5 column, equilibrated with 50 mM HEPES pH 7.3, 10 mM $CaCl_2$ and 1% *n*-octylglucoside. Active fractions, which were eluted by a NaCl gradient (0−300 mM) containing the application buffer, were pooled, concentrated to a total volume of 200 μl by a Centricon 30 microconcentrator, and passed through a second FPLC system consisting of a Sepharose 12 HR 10/30 column equilibrated with 50 mM HEPES, pH 7.3, 10 mM $CaCl_2$, 300 mM NaCl containing 1% *n*-octylglucoside. Furin fractions were concentrated as described above and used for analysis.

Synthesis of peptidyl chloroalkylketones

Alanyl-lysyl-arginyl chloromethylketone (AKR-CMK), tyrosyl-alanyl-lysyl-arginyl chloromethylketone (YAKR-CMK), tyrosyl-alanyl-arginyl-alanyl-lysyl-arginyl chloromethylketone (YARAKR-CMK) were essentially synthesized as described by Kettner and Shaw (1981). The acylated derivates palmitoyl-phenylalanyl-lysyl-arginyl chloroethylketone (palFAKR-CEK), decanoyl-arginyl-glutamyl-lysyl-arginyl chloromethylketone (dec-REKR-CMK), which contained 70% decanoyl-arginyl-glutamyl (γ-methylester)-lysyl-arginyl chloromethylketone, were prepared by related procedures (Wikstrom *et al.*, 1989). All the syntheses of peptidyl chloromethylketones will be described elsewhere (H.Angliker, P.Wikstrom, E.Shaw, W.Brennan and R.S.Fuller, manuscript in preparation). The acylated peptidyl chloroalkylketones were solubilized at 10 mM concentration in dimethylsulfoxide (DMSO), the non-acylated ones in 1 mM HCl as stock solutions and kept at −20°C until usage. Iodination reactions of YARAKR-CMK with ^{125}I were commercially performed by Amersham-Buchler (Braunschweig, Germany) according to the method described by Rauber *et al.* (1988).

Inhibition experiments

For *in vitro* inhibition tests, 1 μl inhibitor solution was added to 9 μl sample and incubated for 60 min at 0°C, except for the peptidyl chloroalkylketones, which were incubated at 37°C for 30 min. To measure the residual protease activity, 5 μl suspension of FPV with uncleaved HA was added and analyzed as described. For *in vivo* inhibition, inhibitors were dispersed by sonification (Branson sonifier, 50 watt, 10 s) mixed in a total volume of 500 μl Dulbecco's medium and added 1 h post-injection to cell cultures. The inhibitors were added to each medium used in the experiments. For HA cleavage analyses, chicken embryo cells were labeled 5 h after infection with [^{35}S]methionine (10 μCi/ml) for a period of 15 h. Viral proteins were immunoprecipitated from the cells and analyzed by SDS–PAGE. Fusion of BHK21-F cells induced by FPV was studied in the absence or presence of peptidyl chloromethylketones at different concentrations added to medium 1 h post-injection. After 6 h, the cells were treated for 2 min with RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) adjusted to pH 5.1 with HCl, which was then replaced by the original medium for 3 h at 37°C. After fixation with ethanol at 0°C for 5 min, cells were washed twice with water and stained for 20 min with 1:10 diluted Giemsa solution (Merck, Darmstadt, Germany). The polykaryons were photographed under a microscope at 200 × magnification.

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