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Processing of viral glycoproteins by the subtilisin-like endoprotease furin and its inhibition by specific peptidylchloroalkylketones

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Summary — The spike glycoproteins of many enveloped viruses are proteolytically cleaved at the carboxytermini of sequences containing the basic motif R-X-K/R-R. Cleavage is often necessary for the fusion capacity of the glycoproteins and, thus, for virus infectivity. Among these viruses are pathogenic avian influenza viruses, human parainfluenza virus, human cytomegalovirus, and human immunodeficiency virus; it has been demonstrated that these viruses can be activated by furin. Indigenous furin has been identified in T-lymphocytes, which are host cells for HIV. Furin has been localized in the TGN and on the surface of cells after vectorial expression. Peptidylchloroalkylketones have been designed that inhibit with high specificity cleavage and fusion activity of viral glycoproteins, as well as virus replication.

viral glycoproteins / proteolytic activation / furin / peptidylchloroalkylketones

Introduction

Endoproteolytic cleavage, usually 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. All these proteins play important roles in a large variety of different biological processes, and their functions depend on proteolytic cleavage of the proteins (for a review see [1, 2]). The same type of processing has also been observed with many viral membrane proteins. In general, cleavage appears to be essential for the function of these proteins in virus entry into the host cells. Cleavage is therefore necessary for infectivity of many enveloped viruses. In some instances, cleavage proved to be a crucial factor in determining organ and host tropism, spread of infection, and pathogenicity [3, 4]. Cleavage often results in the exposition of hydrophobic sequences which induce fusion of viral envelopes with membranes of the target cells, either at the cell surface or in endosomes [5]. Preceding the fusion inducing hydrophobic sequence there is a proteolytic cleavage signal. The majority of viral glycoproteins contains the consensus sequence R-X-K/R-R (table I) which is cleaved by ubiquitous intracellular proteases at the carboxyterminal arginine residue, whereas a minority has a single arginine residue at that site and is cleaved by secreted proteases available in only a few host systems (for a review see [6]). The structures of multibasic cleavage sites have been correlated with the cleavability of several viral glycoproteins which were

Abbreviations: Boc-RVARR-AMC, N-α-t-butyloxycarbonyl-L-arginyl-L-valyl-L-arginyl-L-arginine-7-amido-4-methylcoumarin; decFAKR-CMK, decanoyl-L-phenyl-alanyl-L-alanyl-L-lysyl-L-arginyl-chloromethylketone; decRAIR-CMK, decanoyl-L-arginyl-L-alanyl-L-isoleucinyl-L-arginyl-chloromethylketone; decRAKR-CMK, decanoyl-L-arginyl-L-alanyl-L-lysyl-L-arginyl-chloromethyl-ketone; decREKR-CMK, decanoyl-L-arginyl-L-glutamyl-L-lysyl-L-arginyl-chloromethylketone; decRIKR-CMK, decanoyl-L-arginyl-L-isoleucyl-L-lysyl-L-arginyl-chloromethylketone; decRLKR-CMK, decanoyl-L-arginyl-L-leucyl-L-lysyl-L-arginyl-chloromethylketone; decRRVKR-CMK, decanoyl-L-arginyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone; decRVKR-CMK, decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone; DFP, diisopropyl fluorophosphate; EGTA, ethylene glycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; EDTA, ethylenediamine-tetraacetic acid; E64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; FPV, fowl plague virus; HA, hemagglutinin; moi, multiplicity of infection; pi, post infection; pfu, plaque forming units; PMSF, phenylmethyl-sulfonyl fluoride; TLCK, N-p-Tosyl-L-lysinylchloromethylketone; TPCK, N-Tosyl-L-phenyl-alanyl-chloromethylketone.

Table I. The consensus sequence R-X-K/R-R at the multibasic cleavage sites of viral glycoproteins.

Virus	*Glycoprotein*	*Cleavage site*
Orthomyxoviridae		
A/FPV/Rostock/34 (H7)	HA	KKREKR GL
A/FPV/Dutch/27 (H7)	HA	KKRRKR GL
A/Tern/SA/61 (H5)	HA	TRRQKR GL
Paramyxoviridae		
NDV (Miyadera)	F	GRRQRR FI
Simian virus 5	F	TRRRRR FA
Parainfluenza virus	F	DPRTKR FF
Mumps virus	F	SRRHKR FA
Measles virus	F	SRRHKR FA
Respiratory-syncytial virus	F	KKRKRR FL
Flaviviridae		
Yellow fever virus	M	SGRSRR SV
West Nile virus	M	SRRSRR SL
Togaviridae		
Sindbis virus	E2	SGRSKR SV
Semliki Forest virus	E2	GTRHRR TV
Coronaviridae"		
Infectious bronchitis virus	E2	TRRFRR SI
Mouse hepatitis virus JHM	E2	SRRARR SV
Retroviridae		
HIV 1	env	VQREKR AV
Rous sarcoma virus	env	GIRRKR SV
Friend leukemia virus	env	SYRHKR EP
Herpesviridae		
Human cytomegalovirus	gB	THRTRR ST
Varicella zoster virus	gB	NTRSRR SV
Epstein Barr virus	gB	LRRRRR DA

Data were taken from [6], where the references are cited in.

extensively studied. Among them are the hemagglutinin of pathogenic avian influenza viruses [7–13], the F-protein of Simian virus 5 [14], the F-protein of human parainfluenzavirus type 3 [15], the envelope glycoprotein (gp160) of human immunodeficiency virus (HIV-1) [16–20], the envelope glycoprotein of Rous sarcoma virus [21], and the glycoprotein B (gB) of human cytomegalovirus [22].

The ubiquitous proteases responsible for cleavage at sequences of several arginine and lysine residues were not well understood until recently. It was known that the activating enzyme of the hemagglutinin of the fowl plague virus (FPV), an influenza virus, is membrane bound, calcium dependent, has a neutral pH optimum [23], and can be inhibited by specific peptidylchloroalkylketones [24]. Such enzymes appeared to be highly conserved, since the hemagglutinin of FPV is activated not only in virtually all mammalian and avian cells analyzed but also in invertebrate cells [25]. It was therefore interesting to find that the subtilisin-like protease Kex2 from the yeast *Saccharomyces cerevesiae* resembles these enzymes in its catalytic and other biochemical properties [26]. Kex2 occurs in organelles corresponding to the Golgi apparatus of higher eukaryotic cells [27].

Recently, genes encoding subtilisin-related endoproteases have been isolated which cleave proproteins at multiple basic amino acids (for reviews see references in [28, 29]). Among them is furin, thought to be a promising candidate for the proteolytic processing of viral glycoproteins at multibasic sites. Furin is suggested to be involved in protein processing in the

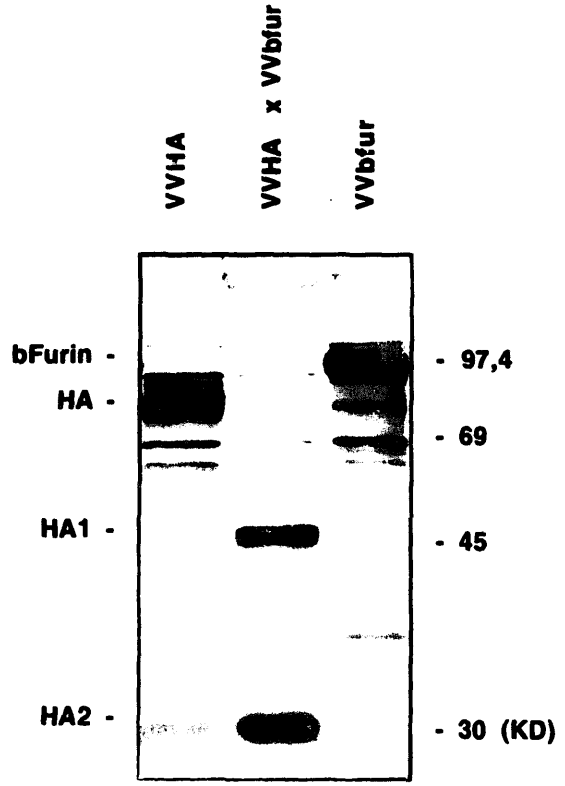

Fig 1. Co-expression of bovine furin and hemagglutinin of fowl plague virus by recombinant vaccinia virus. CV-1 cells were labeled for 1 h with [^{35}S]methionine (100 µCi/ml) at 20 h pi with recombinant vaccinia viruses VV:HA and VV:bfur, expressing the *fur* gene [32] and the HA gene of influenza virus A/FPV/Rostock/34 (H7N), respectively. After 1 h chase with unlabeled methionine, the cells were lysed, the vectorially expressed proteins immunoprecipitated with the antisera, the first two lanes with IgG specific for FPV HA and the third lane with a furin-specific IgG, and analyzed by SDS-PAGE and fluorography as described before [37].

constitutive exocytosis route. Furin has a hydrophobic carboxy-terminal sequence indicating that it is a type I membrane protein. It is calcium-dependent and has a neutral pH optimum. The available evidence indicates that furin requires an arginine in position –4 at its cleavage site, in addition to the paired basic residues in positions –1 and –2. Thus, suitable substrates have tetrabasic sequences (R-K/R-K/R-R) or tribasic sequences (R-X-K/R-R) as found in glycoproteins belonging to various virus families (table I). Like the other subtilisin-like endoproteases furin is activated by a calcium-dependent autocatalytic removal of a pro-sequence at a R-T-K-R cleavage site ([30, 31]; Vey *et al*, submitted). By overexpressing furin in different cell lines, its proteolytic activity could be assayed and it has been shown to activate von Willebrand factor [32, 33], β-nerve growth factor [30], renin [34], and factor C3 [35].

Results and discussion

Viral glycoproteins are cleaved at multibasic cleavage sites by recombinant furin

That viral glycoproteins are activated by furin has been shown by co-expression of recombinant furin and the hemagglutinin of FPV [36] (fig 1), the envelope glycoprotein gp160 of HIV-1 [37], the F-protein of a pathogenic strain of Newcastle disease virus [38], the glycoprotein E2 of Sindbis virus [39], the F-protein of the human parainfluenzavirus [15], and the glycoprotein B of cytomegalovirus [40]. Although it was clear from these studies that furin overexpressed from recombinant vaccinia virus activated viral glycoproteins, it was of interest to see if the indigenous protease activating a virus in a host cell is also furin.

Identification of endogenous furin in bovine kidney cells and T-lymphocytes

Furin has been identified in MDBK cells, a bovine kidney cell line allowing *in vivo* replication of FPV [36]. The nature of the protease activating gp160 of HIV in the target cells, *eg* lymphocytes, has been challenged when a protease distinct from furin was purified from Molt-4 clone 8 cells [41]. Therefore, it was interesting to know whether furin is present in natural host cells of HIV infection. We chose Jurkat cells, another $T4^+$ lymphocyte cell line, to isolate the gp160 activating enzyme(s) by using conventional purification procedures including cell fractionation, sucrose gradient centrifugation and column chromatographies as we described before for MDBK cells [36]. The chromatographic profiles of the enzyme activity through MonoQ and Superose columns were identical to those obtained when furin was expressed in CV-1 (African green monkey) cells by a recombinant vaccinia virus and isolated by the same procedure and to those that were obtained for endogenous furin from MDBK cells [36]. Therefore, it is reasonable to assume that furin is the activating enzyme in lymphocytes also. On the other hand, it cannot be excluded that other proteases than furin may exist in target cells for HIV. Therefore, additional analyses of host cells are needed to see whether furin is virtually the dominant activating enzyme for HIV.

Localization of furin within the exocytotic export route

Previous experiments had suggested that cleavage by furin occurs at a late stage of the exocytosis process, but before delivery of viral glycoproteins to the plasma membrane [42–44]. We have now examined the subcellular localization of vaccinia virus-expressed furin by confocal immunofluorescence microscopy. Using TGN38 as a well established reference protein for the *trans*-Golgi network (TGN), we found that furin resides predominantly in this compartment which is also shown in figure 2A. However, when furin was extremely overexpressed by multicopy transfections of normal rat kidney (NRK) cells with the plasmid pSG5:bFur (Schäfer *et al*, submitted) it also appeared on the cell surface, indicating that under these conditions a certain amount of furin was transported along the constitutive secretory pathway to the plasma membrane (fig 2B). Moreover, we observed that a shortened furin, probably an anchorless form, was secreted into the supernatant of cell cultures overexpressing the enzyme (unpublished results). Similar results have been published by others [45, 46].

Inhibition of viral glycoprotein activation by sequence-specific peptidyl-chloroalkylketones

Peptidyl derivations of chloroalkylketones are potent inhibitors of serine proteases [47]. We have previously shown that, unlike peptidylchloroalkylketones containing a single arginine, such compounds with paired basic amino acids, *ie* R-R or K-R, inhibited cleavage activation of the FPV hemagglutinin and thus prevented multiple cycles of virus replication [24]. Since it became clear that the consensus sequence has an additional arginine at –4 of the cleavage site [13, 48], peptidylchloroalkylketones with an arginine in this position have been synthesized and tested for their inhibitory effect on cleavage. The amino termini were acylated to deliver the peptide inhibitors to intracellular target membranes. Acylated inhibitors were, indeed, more efficient than those synthesized without this modification [24]. Peptidylchloroalkylketones

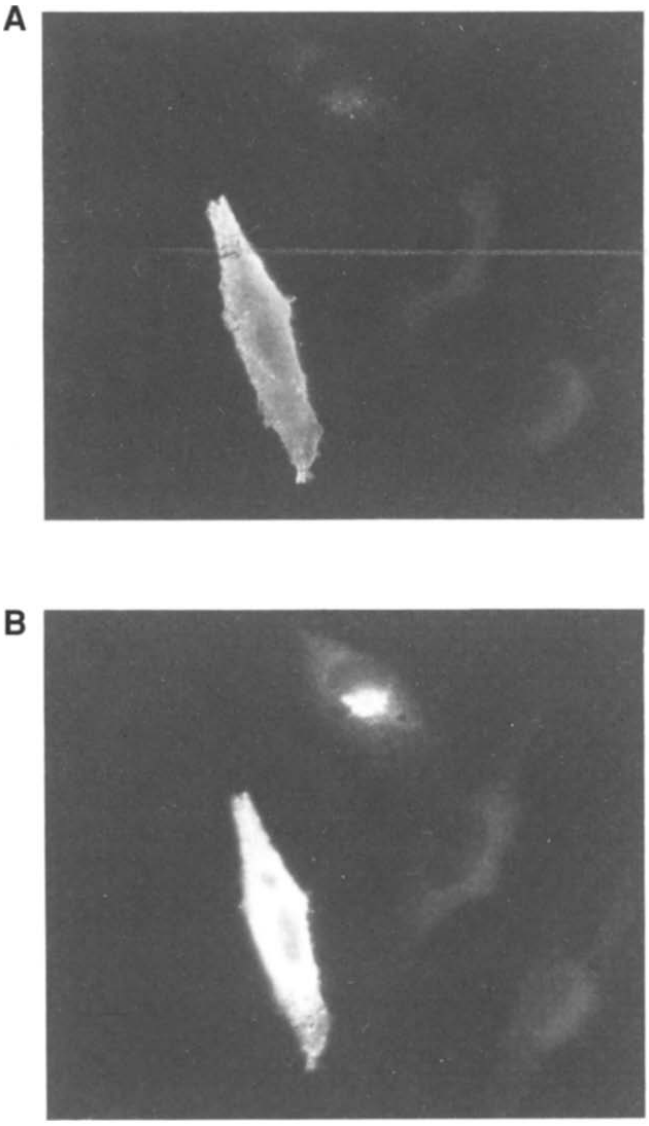

Fig 2. Localization of furin. After expression of pSG5:fur for 6 h in normal rat kidney (NRK) cells grown on coverslips, cells were fixed by paraformaldehyde, kept without detergent (**A**) or treated with 0.1% saponin (**B**). Cells were processed for double immunofluorescence analysis using antibodies first specific for the furin ectodomain (**A**) after permeabilization specific for the intracellular domain of furin (**B**). Cells were analyzed under an Axiophot microscope (Zeiss) at x 200 magnification.

containing the common structure acyl-R-X-K/R-R-CMK proved to be potent inhibitors for cleavage of FPV HA [36] and gp160 of HIV [37] (fig 3), F-protein of human parainfluenza virus type 3 [15] (fig 4), and gB of cytomegalovirus [40]. Figure 4 shows also that only tri- and tetrabasic but not dibasic sequences containing arginine at position –1 and lysine at position –2 of the cleavage site of the parainfluenza F-protein were recognized by furin.

It was of interest to find out if the inhibitory effect of the furin-specific peptidylchloroalkylketones could be altered by modifying the non-essential residues in the peptide sequence. The substrate homologous inhibitors have been modified to optimize their inhibitory effect on virus activation. As seen in table I, the amino acid at position –3 of the furin motif is rather variable. Therefore, various amino acids were placed in this position and tested for their inhibitory efficiency using HIV gp160 as substrate. The cleavage was not much changed when glutamic acid was exchanged for alanine [36, 37], valine (fig 3), or isoleucine, and decreased slightly when leucine was present in this position. Extension of the furin motif to two pairs of basic amino acids, as found in decRRVKR-CMK, did not increase the inhibitory potential either (fig 5). Other modifications such as the replacement of methyl by ethyl within the chloroalkyl group or the exchange of decanoic by palmitic acid, have been proved to be without any effect [24, 36, 37]. Chloroalkylketones are unstable under physiological conditions with a half-life of about 4 to 8 h (data not shown). Resistance to proteolytic degradation may be achieved, for instance, by the introduction of pseudopeptide bonds and might result in higher stability.

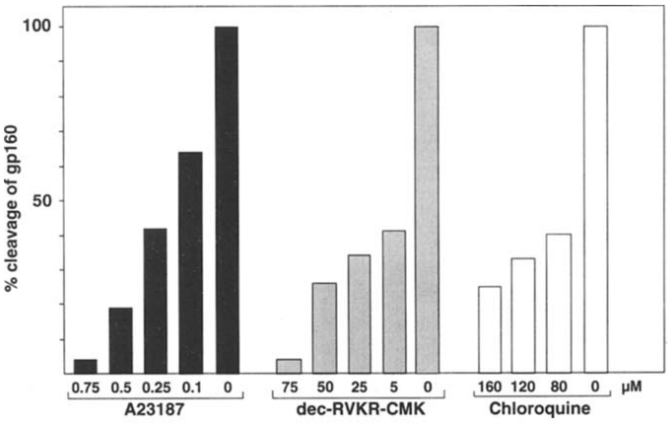

Fig 3. Effects of various inhibitors on furin-mediated cleavage of the HIV glycoprotein. Jurkat cells were infected with HIV gp160 recombinant vaccinia virus at a moi of 5 pfu/cell. The ionophore A23187 was added in the absence of calcium, dec-RVKR-CMK [58] and chloroquine in the presence of calcium to the medium 5 h pi. The lymphocytes were labeled with [^{35}S]methionine (100 μCi/ml) at 6 h pi for 2 h. After 4 h chase with unlabeled methionine the vectorially expressed HIV proteins were immunoprecipitated and the degree of cleavage was analyzed by SDS-PAGE and fluorography as described [37].

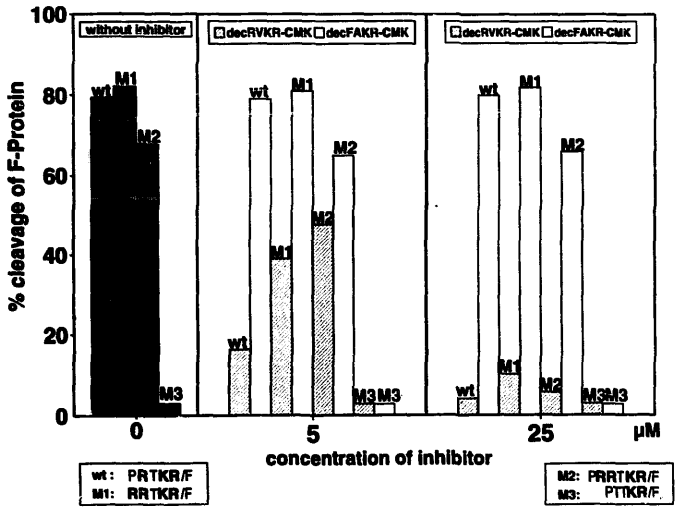

Fig 4. Effects of basic peptidylchloroalkylketones on the cleavage of parainfluenza virus F-protein, wild type and mutants. CV-1 cells were infected with recombinant vaccinia virus containing the F-protein of the human parainfluenza virus type 3 wild type (wt) and three mutants (M1, M2, and M3). The mutants differ from wild type at the cleavage site as indicated [15]. Cells expressing the F-protein were treated with the inhibitors decRVKR-CMK and decFAKR-CMK [58] at final concentrations of 5 and 25 µM. At 6 h pi, proteins were labeled with [^{35}S]methionine (100 µCi/ml) for 1 h and the radioactive label chased by non-radioactive methionine (final concentration 10 mM) for 3 h. Immunoprecipitated proteins were analyzed by SDS-PAGE followed by fluorography and quantification [15, 37].

Peptidylchloroalkylketones react with furin without inhibiting the intracellular transport of viral glycoproteins

The finding that radioactively labeled [^{125}I]YARAKR-CMK was covalently bound to furin indicated that furin is the target molecule for this inhibitor [36]. But it remained to be seen if the inhibition had an effect on transport of viral glycoproteins to the cell surface, where virus budding of most enveloped viruses occurs. This problem was studied by expression of gp160 recombinant vaccinia virus in HeLa-T4 cells. In the presence of decREKR-CMK, mature glycosylated gp160 was immunoprecipitated from the cell surface in an endoglycosidase H resistant form (data not shown), whereas only the cleavage product gp120 was immunoprecipitated from the plasma membrane in the absence of the inhibitor (fig 6). This indicated that the uncleaved glycoprotein of HIV was still transported to the cell surface when furin activity was abolished. This observation agreed with the fact that virions with uncleaved gp160 were released from HIV infected cells in the presence of decREKR-CMK [37].

Furthermore, the HIV glycoprotein which is normally shedded as soluble gp120 from the cell surface into the medium was not observed in supernatants of cells treated with this inhibitor (fig 6). This is an interesting aspect, because soluble gp120 has been thought to play an important role in the HIV pathogenesis [49–51]. It will be interesting to see if the effect of inhibition on gp120 shedding can be used for therapeutical purposes.

Furin inhibition suppresses the biological function of viral glycoproteins

Cell-cell fusion mediated by the proteolytically activated glycoprotein of HIV was demonstrated by co-expression of gp160 and furin. Late after vaccinia virus infection it was shown that endogenous furin activity disappeared in cells [30, 36, 37]. Therefore, single expression of the HIV glycoprotein yielded the uncleaved form and no fusion occurred between CV-1 cells and HeLa-CD4 cells which are susceptible to fusion by the viral glycoprotein, since HeLa-CD4 cells contain the virus receptor. However, syncytia formation was clearly observed when furin and gp160 were co-expressed in CV-1 cells (fig 7). To analyze the effects of the chloroalkylketones on the fusion activity of the envelope glycoprotein, syncytia formation has been assayed in HeLa-CD4 cells. The data showed that inhibitors with the correct consensus sequence (decRAKR-CMK and decREKR-CMK) blocked fusion, whereas inhibitors deviating from that sequence motif (decRAIR-CMK and decFAKR-

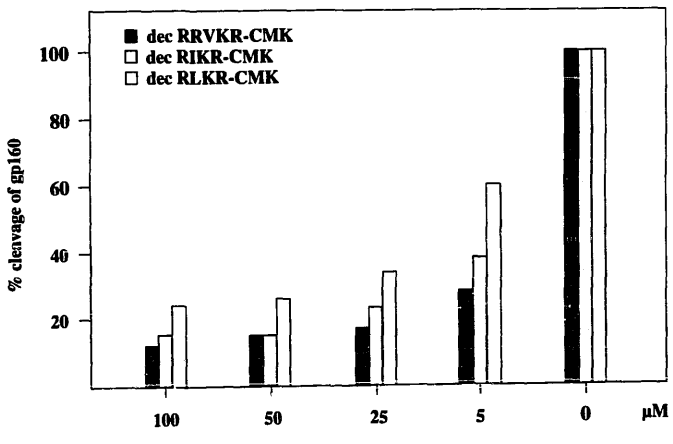

Fig 5. Effect of various peptidylchloroalkylketones on cleavage of HIV gp160. HeLa-CD4 cells were infected with HIV gp160 recombinant vaccinia virus, treated with three different inhibitors [58] at concentrations as indicated, and labeled with [^{35}S]methionine. The immunoprecipitated HIV glycoprotein was analyzed as described in legend to figure 3 [37].

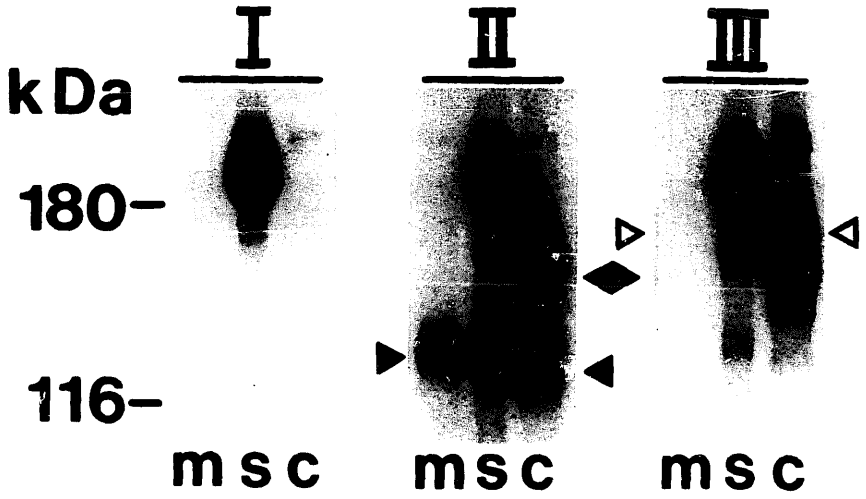

Fig 6. Cell surface expression of the HIV glycoprotein in the presence and the absence of a furin-specific peptidyl-chloralkylketone. HeLa CD4 cells were infected with wild type (I) or HIV gp160 recombinant (II, III) vaccinia viruses at a moi of 3 pfu per cell, treated with decREKR-CMK at a final concentration of 50 μM, and labeled 5 h pi for 2 h with [^{35}S]methionine (100 μCi/ml) which was then chased for 4 h by addition of non-radioactive methionine. HIV glycoprotein was immunoprecipitated from the medium (m), the cell surface (s), and the lysed cells (c) and analyzed by SDS-PAGE and fluorography. The positions of gp160 with fully processed carbohydrate side chains are indicated by an open triangle, gp160 with high-mannose carbohydrate side chains by a filled rhombus, gp120 with fully processed carbohydrate side chains by a filled triangle.

CMK) did not inhibit fusion at micromolar concentrations [37]. The interesting question of whether furin specific peptidyl-chloroalkylketones prevent replication of viruses which are activated by furin has also been examined in studies with FPV [24, 52] and HIV-1 [37]. The data reviewed in table II were taken from previous experiments [37] and show that the inhibitor decREKR-CMK containing the authentic cleavage site sequence of gp160 of HIV-1 and the inhibitor decRAIR-CMK which rather inefficiently inhibits the furin cleavage released infectious HIV from transformed T-lymphocytes (MT-4 cells). Further, virus release decreased with increasing concentrations of both inhibitors added to the cell cultures, probably due to the toxicity of such inhibitors in cell cultures at high concentrations. But cell cultures treated with the decRAIR-CMK released fewer virus particles due to the higher toxicity of this inhibitor. However, the infectivity of virus particles propagated in the presence of decREKR-CMK were significantly lower than that of virus grown in the presence of decRAIR-CMK. Interestingly, virus particles which were from cells treated with decREKR-CMK contained gp160 again indicating that cleavage is not necessary for the transport of the viral glycoprotein to the cell surface and incorporation into virions. The reduced infectivity of virus produced in the presence of decREKR-CMK is clearly a consequence of the inability of gp160 to induce fusion as demonstrated before on gp160 with a mutated cleavage site [16]. Similar results were confirmed with other peptidylchloroalkylketones which effectively inhibit the furin cleavage (Hallenberger, unpublished results).

Table II. Infectivity of HIV-1 released from inhibitor treated lymphocytes (MT-4 cells).

Concentration of inhibitor (μM)	*Virus release[a] (%)*	*Infectivity[b] (%)*
decREKR-CMK		
10	85	31
25	76	14
decRAIR-CMK		
10	80	90
25	32	70

Data taken from [37]. [a]HIV-1 particles, produced from cell cultures were quantitated by ELISA. The amount of virus released from untreated culture was taken as 100%. Lower yield of released virus displays toxic effects of the inhibitors. [b]Equal amounts of virus were employed to infect fresh MT-4 cells and infectivity was measured again by ELISA. The infectivity of virus released from the untreated cell cultures was taken as 100%.

Other agents inhibiting furin-mediated cleavage of viral glycoproteins

The ionophore A23187 [23], as well as the chelating agents EGTA and EDTA prevent cleavage of FPV HA, reflecting the calcium dependence of the HA activating enzyme that turned out to be furin [36]. Other inhibitors specific for metalloproteases, such as phenanthroline, were ineffective. Of the inhibitors specific for cysteine proteases, such as iodoacetamide,

N-ethylmaleimide, cobalt chloride, cadmium acetate, E64, and mercuribenzoate, only the last one was effective to some degree. The serine protease inhibitors DFP, PMSF, TLCK, TPCK, leupeptin, and aprotinin did not influence cleavage, nor did pepstatin, an inhibitor of aspartate proteinases [36].

Following the lines of previous experiments in which FPV containing uncleaved HA was obtained in the presence of the ionophore A23187 [23], we investigated whether A23187 also prevents the cleavage of gp160 of HIV. Less than 10% of gp160 was cleaved, when it was expressed from recombinant vaccinia virus in Jurkat cells in the presence of 1 μM A23187 (fig 3). Similar results were obtained with HIV infected cells [53]. Chloroquine, an agent raising the pH in intracellular acidic compartments has been shown to inhibit cleavage of FPV HA [54] and has also an inhibitory effect on cleavage of gp160 (fig 3). The exact mechanism how chloroquine reduces furin activity is not yet clear. Brefeldin A, a fungal metabolite that has profound and dramatic effects on the secretory pathway in mammalian cells (for review and references see [55]), totally inhibits cleavage of vaccinia virus expressed gp160 when added to the medium at 2 μM concentrations (data not shown). A likely explanation for this effect would be a block in gp160 transport to the TGN. Alternatively, mutated turkey ovomucoid containing the consensus optimal

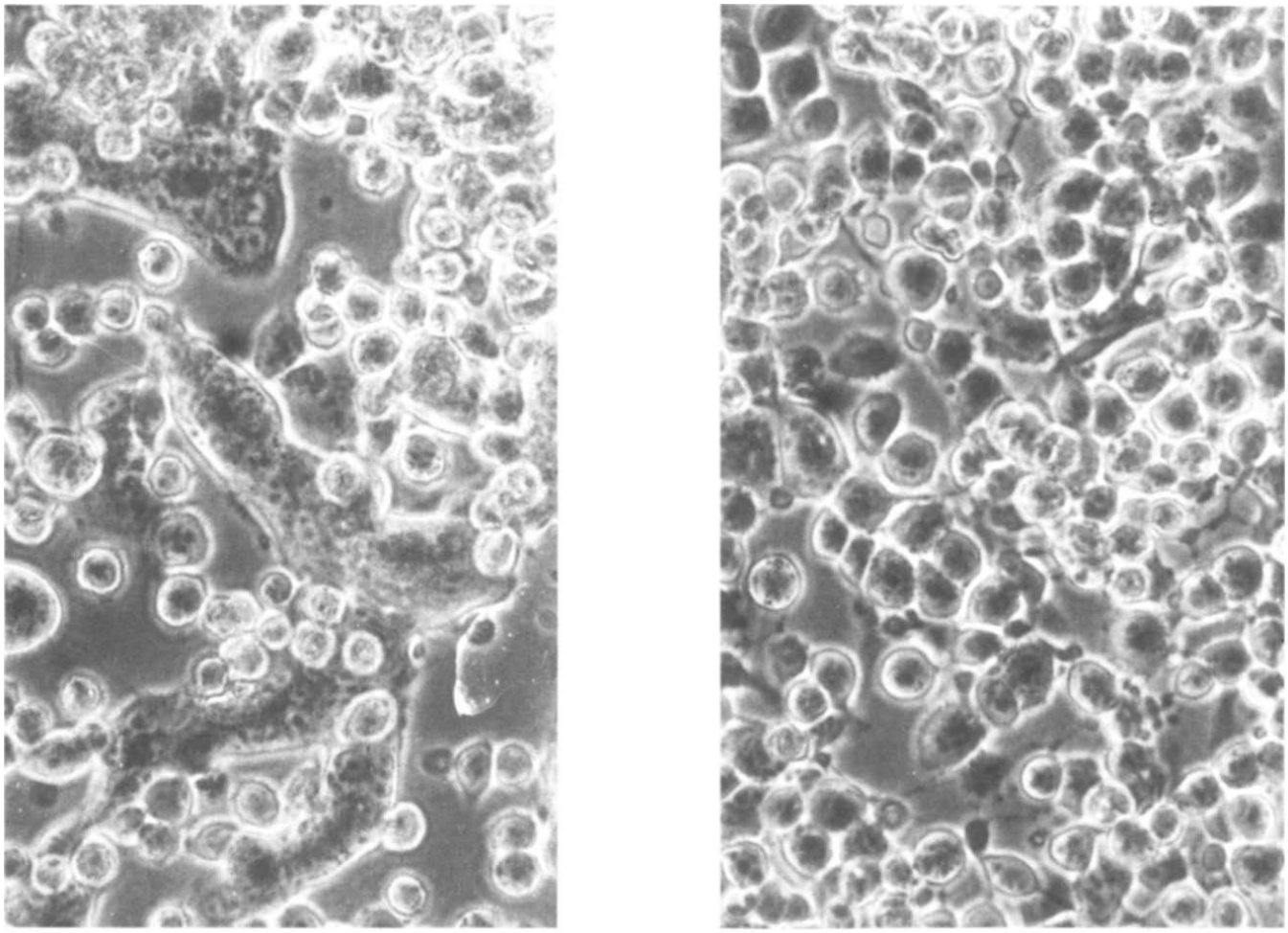

Fig 7. Fusion assay. HIV gp160 recombinant and furin recombinant vaccinia viruses were co-expressed (**A**) and HIV gp160 expressed alone (**B**) in CV-1 cells. At 23 h pi the infected cell cultures were overlaid with HeLa-CD4 cells, and incubated at 37°C for 1.5 h. Fusion activity was microscopically analyzed. Only the HeLa-CD4 cells were focused under the microscope and are shown at x 150 magnification.

sequence R-X-K-R [56] and mutated α_1-antitrypsin containing the minimal sequence R-X-X-R [58] required for efficient processing by furin were proved to be efficient inhibitors of human furin, of which the latter inhibited HIV-1 gp160-dependent membrane fusion [57].

Conclusions

From a virologist's point of view, furin is of particular interest as a cellular endoprotease responsible for the cleavage activation of the surface glycoproteins of a whole series of important pathogens, although other enzymes, such as PACE4, PC5/PC6, or even PC1/PC3 and PC2, have similar substrate specificities and are considered as potential activating endoproteases for viral glycoproteins. Cleavage of the glycoproteins is essential for the ability of the viruses to enter cells and, thus, for their infectivity. Furin inhibitors, such as peptidylchloroalkylketones of which the peptidyl moiety is homologous to the cleavage site R-X-K/R-R, prevent cleavage and fusion activity of viral glycoproteins and, as a consequence, virus infectivity, as has been shown for a pathogenic avian influenza virus and HIV-1. In addition, by inhibiting cleavage of the HIV glycoprotein shedding of gp120 subunits from cells and virions is reduced which is believed to play an important role in the pathogenicity of this virus. Our data indicate that it may be possible to develop improved inhibitors, especially substances which are more stable under physiological conditions. It remains to be seen in future studies if inhibitors can be designed with low toxicity that are useful as antiviral substances.

Acknowledgments

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