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Inhibition of Proteolytic Activation of Influenza Virus Hemagglutinin by Specific Peptidyl Chloroalkyl Ketones

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Lyases of cultured cells have been analyzed for arginine-specific endoproteases using peptidyl-p-nitroanilides as chromogenic substrates. The enzymes present in MDBK, MDCK, VERO, BHK, and chick embryo cells required lysine–arginine or arginine–arginine pairs as cleavage sites, whereas chorioallantoic membrane cells contained, in addition, an activity that could cleave at a single arginine. The effect of peptidyl chloroalkyl ketones on the activation of the fowl plague virus hemagglutinin by the proteases specific for paired basic residues has been investigated. When virions containing uncleaved hemagglutinin were incubated with lysates of uninfected cells, cleavage was completely inhibited by peptidyl chloroalkyl ketones containing paired basic residues at a concentration of 1 mM. In contrast, a compound containing a single arginine had no inhibitory activity. When dibasic peptidyl chloroalkyl ketones were added to infected cell cultures, cleavage of hemagglutinin and multiple cycles of virus replication were inhibited at 10 mM. However, a 100- to 200-fold increase of the inhibitory activity in intact cells could be achieved by N-terminal acylation. These studies suggest a potential role of peptidyl chloroalkyl ketones as antiviral agents.

INTRODUCTION

Biosynthesis of the hemagglutinin of influenza virus involves post-translational proteolytic cleavage of the precursor HA into the subunits HA1 and HA2. Cleavage is necessary for the expression of the fusion capacity of the hemagglutinin and, thus, for initiation of infection (Klenk et al., 1975; Lazarowitz and Choppin, 1975). Two host enzymes are involved in cleavage: first a “trypsin-like” endoprotease acts on a distinct arginine–glycine bond. Subsequently, a carboxypeptidase eliminates the basic amino acids at the cleavage site. Proteolytic activation proved to be of high importance for the spread of infection and for pathogenicity. The hemagglutinins of mammalian and of pathogenic avian influenza viruses which cause local infections are cleaved only in a restricted number of cell types. In contrast, the hemagglutinins of pathogenic avian influenza viruses which cause systemic infection are activated in a broad range of different host cells. Important determinants for these differences in proteolytic activation are the structure of the cleavage site on the hemagglutinin and the specificity of the endoproteases present in individual cells. The hemagglutinin of most apathogenic avian and of mammalian influenza viruses have a single arginine residue at the cleavage site which is recognized by enzymes present only in few cells, whereas the hemagglutinins of the pathogenic avian influenza viruses have a cleavage site consisting of several basic amino acids which can be cleaved by endoproteases present in many cells (for review see Klenk and Rott, 1988).

Post-translational proteolytic cleavage at multiple basic residues appears to be a general principle by which many hormones, enzymes, and other biologically important peptides or proteins are activated (Docherty et al., 1982; Herbert and Uhler, 1982). This type of cleavage has been also observed with a whole series of other viral glycoproteins. These include the F protein of paramyxoviruses (Scheid and Choppin, 1974; Paterson et al., 1984), the precursor to the E2 protein of coronaviruses (Binns et al., 1985), the surface glycoprotein of arenaviruses (Romanowski et al., 1985), and the env protein of retroviruses (Shinnick et al., 1981). The minimal consensus sequence that can serve as a recognition signal for the converting enzymes appears to be a pair of basic amino acids, most frequently lysine–arginine or arginine arginine. However, the length of the basic peptide at the cleavage site often exceeds these 2 amino acids, and the available evidence indicates that the additional basic residues may increase cleavability (Kawaoaka et al., 1988) and pathogenicity (Ohuchi et al., 1989).

Peptidyl chloroalkyl ketones are potent protease inhibitors, that covalently label the substrate binding sites of proteolytic enzymes. Considerable specificity...
for inactivating “trypsin-like” enzymes involved in coagulation and fibrinolysis has been obtained by modifying the peptidyl moiety of the reagents (Kettner and Shaw, 1981). In the present study we have analyzed the effect of various peptidyl chloroalkyl ketones on hemagglutinin cleavage and replication of a pathogenic avian influenza virus, fowl plague virus. We show that compounds containing paired basic amino acids are effective inhibitors.

MATERIALS AND METHODS

Virus growth

The influenza virus strains A/FPV/Rostock/34 (H7N1) and A/FPV/Dutch/27 (H7N7) (Dobson variant, adapted to growth in BHK cells) were used. Eggs were propagated in the allantoic cavity of 11-day-old embryonated eggs. The Dutch strain was grown in BHK21 F cells in the presence of ionophore A23187 (Calbiochem, Frankfurt) added to reinforced Eagle’s medium (REM) lacking calcium chloride, when virus with uncleaved hemagglutinin was needed (Klenk et al., 1984). The Rostock strain was grown in confluent monolayers of chicken embryo cells (CEC). To obtain radioactive virus particles, [35S]methionine (20 µCi/ml) or [3H]glucosamine (5 µCi/ml) was added to methionine-free medium or to medium with glucose replaced by 10 mM fructose, respectively. Before metabolic labeling with [35S]methionine, cells were depleted of methionine for 30 min. Virus released into the supernatant of the cell cultures was collected by centrifugation and purified on sucrose gradients (Klenk et al., 1977).

When virus was propagated in the presence of peptidyl chloroalkyl ketones, inhibitors were solubilized in 1 mM HCl, except for the palmitoylated one, which was dissolved in dimethyl sulfoxide. The stock solutions of the peptidyl chloroalkyl ketones, which were stored at −20°C, were diluted 10-fold with PBS or REM just before use. When the palmitoylated inhibitor was added to medium it was dispersed by ultrasonification in a Branson Sonifier (10 sec, 50 W).

Virus assays

Hemagglutination and plaque assays were carried out for quantification of the virus according to standard procedures.

Analysis of the viral glycoproteins

Samples containing radioactively labeled viral glycoproteins were analyzed by SDS–PAGE (Laemmli, 1970) directly or after immunoprecipitation. Gels contained 12% acrylamide and 0.32% bisacrylamide. After treating gels with Enhance (New England Nuclear), fluorography were carried out on Kodak X-Omat AR films at −70°C. Nonradioactive viral proteins were analyzed by immunoblotting (Kuroda et al., 1986). For immunoblotting and immunoprecipitation a rabbit antisera against FPV (Rostock strain) was used.

Cell lysates

Monolayer cultures of CEC, chorioallantoic membrane (CAM) cells (Klenk et al., 1975), and various other cell types were scraped from petri dishes (5-cm diameter) and resuspended in 1 ml PBS, containing 10 mM CaCl2 and 2% n-octylglucoside. The cells were sonicated with a Branson Sonifier for 3 sec at 0°C and immediately used for assays (Klenk et al., 1984).

Assays for proteases

The following chromogenic substrates, which were purchased from Bachem (Bubendorf, Switzerland), were used: N-Cbz-L-tyrosyl-L-lysyl-L-arginine-p-nitroanilide-2TFA (Cbz-YKR-pNA), N-Cbz-L-arginyl-L-arginine-p-nitroanilide-2HCl (Cbz-RR-pNA), and N-Cbz-L-arginine-p-nitroanilide (Cbz-R-pNA). Aqueous solutions of these substrates were added to 50 µl cell lysates in a total volume of 500 µl, containing 10 mM CaCl2. After incubation at 37°C for 2 hr the reaction mixture was centrifuged at 10,000 g for 5 min, and the optical density of the supernatant was measured at 405 nm. When hemagglutinin was used as substrate, about 200 HAU of radioactively labeled FPV (Dutch strain) containing uncleaved hemagglutinin were incubated in 25 µl PBS with 10 µl cell lysates in the presence of 10 mM CaCl2 (Klenk et al., 1984). Proteolytic cleavage was stopped by adding 35 µl sample buffer, containing 1% SDS and 1% dithiothreitol. For electrophoresis, samples were boiled for 2 min and applied to slab gels. Proportions of uncleaved and proteolytically cleaved hemagglutinin before and after protease treatment were estimated by scanning fluorographies on a Bio-Rad Model 620 Videodensitometer.

Synthesis of peptidyl chloroalkyl ketones

Alanyl-lysyl-arginy1 chloromethyl ketone (AKR-CMK), tyrosyl-alanyl-lysyl-arginy1 chloromethyl ketone (YAKR-CMK), phenylalanyl-alanyl-lysyl-arginy1 chloromethyl ketone (FAKR-CMK), and alanyl-phenylalanyl-arginy1 chloromethyl ketone (AFR-CMK) were synthesized as described by Kettner and Shaw (1981). Phenylalanyl-alanyl-lysyl-arginy1 chloroethyl ketone (pAFR-CEK) and palmitoyl-phenylalanyl-alanyl-lysyl-arginy1 chloroethyl ketone (palpAFR-CEK) were prepared by related procedures (Wikstrom et al., 1989).
TABLE 1

TRYPSIN-LIKE PROTEASES WITH DIFFERENT SUBSTRATE SPECIFICITIES
PRESENT IN VARIOUS CELLS

| Cells | Cbz-YKR-pNA | Cbz-RR-pNA | Cbz-R-pNA |
|-------|-------------|------------|------------|
| CAM   | 90          | 99         | 22         |
| CEC   | 80          | 75         | 0.2        |
| MDBK  | 94          | 98         | 0.4        |
| MDCK  | 62          | 51         | 1.1        |
| VERO  | 31          | 43         | 0          |
| BHK   | 15          | 10         | 1.8        |

*Peptidyl-p-nitroanilides (2 mM) were incubated with cell lysates in PBS containing 10 mM CaCl₂ and 2% n-octylglucoside at 37°C for 2 hr. Extinction of p-nitroaniline was measured at 405 nm. The values indicate the percentage of cleavage relative to total cleavage as obtained after incubation of substrate with trypsin (1 μg/ml).

RESULTS

Chromogenic peptides as substrates for endoproteases activating the FPV hemagglutinin

To characterize the proteases responsible for the activation of the FPV hemagglutinin we have used virus with the uncleaved glycoprotein as substrate in previous work. Such virus, which is grown in the presence of the calcium-specific ionophore A 23187 (Klenk et al., 1984), can only be obtained in small amounts. Furthermore, the cell lysates employed in these studies are likely to contain a wide spectrum of different proteases, and it is known that the influenza virus hemagglutinin can be cleaved into fragments similar in size to HA₁ and HA₂ by several nonactivating enzymes that differ in their substrate specificities from the trypsin-like proteases (Garten et al., 1981). For these reasons we have used, in addition to virus grown in the presence of A 23187, chromogenic peptides mimicking the cleavage site as an alternate substrate in the present study.

As shown in Table 1, peptidyl p-nitroanilides containing two basic amino acids are cleaved by lysates obtained from all cells analyzed. There are differences in the relative cleaving activities of the individual cell lines with MDBK and BHK cells on the extreme ends. Variation in the sequence of the two basic residues (arginine-arginine versus lysine-arginine) (Table 1) or N-terminal elongation of the peptide by adding several uncharged amino acids (alanine or tyrosine) (data not shown) had no significant effect on the substrate specificity. However, when the basic sequence was reduced to a single arginine, the peptide had lost its function as a substrate with most cells, except for CAM cells. Thus, the chromogenic peptides resemble very closely in their differential substrate specificities the influenza virus hemagglutinins among which those containing a single arginine are also cleaved only in CAM cells, whereas those containing several basic residues are usually cleaved in many cells. The chromogenic peptides are therefore suitable substrates for the proteases activating the hemagglutinin.

Protease inhibition by peptidyl chloroalkyl ketones in cell lysates

It has been shown that peptidyl chloroalkyl ketones with amino acid sequences analogous to physiological substrates specifically inactivate the corresponding eurine proteases (Kettner and Shaw, 1981). We have therefore used such substances to inhibit the proteases activating the hemagglutinin. Table 2 shows an experiment in which a cell lysate has been first incubated with AKR-CMK as an inhibitor and subsequently with chromogenic substrates containing also two basic residues at the cleavage site. The data indicate, that at inhibitor concentrations of 0.1 and 1 mM the proteolytic activity is inhibited by 50 and 100%, respectively.

We have then used hemagglutinin as substrate to analyze the effect of various peptidyl chloroalkyl ketones on proteases in cell lysates. Table 3 shows that all compounds including a palmitoylated derivative had inhibitory activity if they contained a pair of basic residues. In contrast, the ketone with a single arginine was ineffective. This observation indicates that the specificity of these inhibitors varies depending on the number of basic residues, as is the case with the substrate specificity of the proteases they interfere with.
TABLE 3

EFFECT OF VARIOUS PEPTIDYL CHLOROALKYL KETONES ON PROTEOLYTIC CLEAVAGE OF HEMAGGLUTININ IN VITRO

| Inhibitor      | Concentration (mM) | Inhibition (%) |
|----------------|--------------------|----------------|
| AKR-CMK        | 1                  | 100            |
| YAKR-CMK       | 1                  | 100            |
| FAKR-CMK       | 1                  | 100            |
| pal-FAKR-CEK   | 0.5                | 100            |
|                | 0.1                | 75             |
|                | 0.01               | 50             |
|                | 0.001              | 12             |
| FAFR-CMK       | 2                  | 10             |

Note. Purified FPV (Dobson variant) with uncleaved hemagglutinin which has been grown in BHK cells in the presence of A23187 (0.25 μM) and [3H]glucosamine (2 μCi/ml) was incubated with MDBK cell lysates as described under Materials and Methods. Before incubation with the virus, cell lysates have been pretreated at room temperature for 60 min with various concentrations of the inhibitors indicated. The water-soluble inhibitors were dissolved in 1 mM HCl, the acylated inhibitor in dimethyl sulfoxide. Thereafter the virions were disintegrated in sample buffer under reducing conditions. Viral proteins were analyzed by SDS–PAGE and fluorography. Cleaved and uncleaved hemagglutinin were quantitated by densitometry. The difference in the amount of uncleaved hemagglutinin found in untreated virus and in virus exposed to cell lysate in the absence of inhibitors was considered 100% cleavage.

The effect of peptidyl chloroalkyl ketone on hemagglutinin activation and spread of infection in cell culture

It was now of interest to find out whether peptidyl chloroalkyl ketones are able to interfere with hemagglutinin processing in the course of virus replication in the intact cell. After pretreatment with FAKR-CMK for 24 hr, monolayers of CE cells were infected with FPV under single replication cycle conditions. Virus released from the cultures was purified and analyzed for its polypeptide composition (Fig. 1). Essentially the same results have been obtained, when the Rostock strain, as shown here, and the Dutch strain (data not shown) have been used. When the inhibitor was present before and after infection, virus with uncleaved hemagglutinin was produced. To obtain complete inhibition of cleavage, inhibitor concentrations of 10 mM were needed (Fig. 1b). When the inhibitor was removed from the cell culture after the pretreatment, virus was produced with cleaved hemagglutinin (Fig. 1a) as was the case in the control experiment performed without inhibitor (Fig. 1c). Incubation with inhibitor did not result in a significant reduction of virus release as measured by hemagglutination titers in the media. Even extended inhibitor pretreatment for as much as 3 days had little effect on the cells. These observations indicate that FAKR-CMK prevents cleavage not only in a cell lysate, but also in the infected intact cell. Furthermore, FAKR-CMK showed little toxicity in cell culture. The monobasic inhibitor FAR-CMK, in contrast, proved to be quite toxic. Already at a concentration of 1 mM, cells were severely damaged and did no longer allow virus replication.

The intracellular compartmentalization and the membrane anchorage of the cleavage enzyme (Klenk et al., 1984) may explain the relatively high concentrations of the water soluble inhibitors (10 mM) that are needed to prevent hemagglutinin cleavage in cell culture. Because of its amphophilic character, the acylated compound palFAKR-CEK should have better enzyme access in the intact cell. As has been pointed out above (Table 3), this inhibitor had to be used in similar amounts as the water soluble compounds to be effective in cell lysates. However, when added to cells infected with the Rostock strain, palFAKR-CEK caused total inhibition of cleavage already at a concentration of 50 μM (Fig. 2a), whereas 10 mM were required with FAKR-CMK (Fig. 1). Even at the 5-μM concentration of palFAKR-CEK, partial inhibition was still observed (Fig. 2b). Similar results have been obtained with the Dutch strain (data not shown). Thus, it is clear that acylation enhanced the inhibitory effect in intact cells.

![Fig. 1. Effect of phenylalanyl-alanyl-lysyl-arginine chloromethyl ketone (FAKR-CMK) on biosynthesis and processing of FPV hemagglutinin. The inhibitor FAKR-CMK was dissolved in 1 mM HCl and added in REM at a final concentration of 10 mM to monolayers of chicken embryo cells (a and b). After preincubation with the inhibitor for 24 hr, the cell cultures were infected with the Rostock strain of FPV (10^6 PFU/ml). Virus was propagated in the absence (a) or in the presence of 10 mM FAKR-CMK (b) for a further 24 hr. Virus released into the medium was pelleted by centrifugation, and viral proteins were analyzed by the Western blot technique. An infected cell culture not exposed to the inhibitor is shown as a control (c).](image-url)
Since inhibition of hemagglutinin cleavage has been accomplished, it was of interest to find out whether the compounds interfere with the spread of virus infection. Virus was therefore propagated in CE cell cultures under conditions of multiple cycle replication, and the effect of the inhibitors on virus growth was analyzed. Again, essentially the same results have been obtained with the Roestock and the Dutch strain. As shown in Fig. 3a, FAKR-CMK had to be added to the culture at a concentration of 10 mM to obtain a significant effect. Again, the efficiency of the inhibitor could not be altered by replacing the methyl by an ethyl group, or by adding uncharged amino acids to the amino-terminal end of the peptide (data not shown). However, acylation of the peptide resulted in a distinct increase in inhibitory activity (Fig. 3b). The observation that virus production was only retarded, but not completely suppressed, even under the most effective inhibitory conditions, is most likely explained by gradual degradation of the peptidyl chloroalkyl ketones in cell culture.

**DISCUSSION**

We demonstrate here that dibasic peptidyl chloroalkyl ketones inhibit cleavage activation of the FPV hemagglutinin. These results have been obtained by analyzing hemagglutinin processing, first in CE cell cultures infected with the Roestock and the Dutch strain of this virus, and second, in an *in vitro* system using lysates of MDBK cells and virions of the Dutch strain containing the uncleaved hemagglutinin precursor (Klenk et al., 1984). Since the hemagglutinin has essentially the same cleavage site in the Roestock and in the Dutch strain (Klenk and Rott, 1988), it is legitimate to compare the results obtained in both systems. Specific peptidyl chloroalkyl ketones have been shown in previous studies to be useful agents for the selective inactivation and characterization of viral (Korant et al., 1979) and cellular cysteine and serine proteinases. The latter group of proteinases includes trypsin, plasmin, aprotinin, and several bacterial proteinases, which under certain conditions may also serve as activating enzymes (Lazarowitz et al., 1973; Klenk et al., 1977; Garten et al., 1981; Tashiro et al., 1987a,b). It has been reported that amino caproic acid and aprotinin interfere with activation of the influenza virus hemagglutinin by plasmin present in the alveolar fluid (Zhirnov et al., 1982a,b), and leupeptin inhibits proteinases secreted by infecting bacteria (Tashiro et al., 1987a,b). In contrast to these inhibitors that act in the extracellular space, the peptidyl chloroalkyl ketones, as used in the present study, block intracellular enzymes.

The observation that the palmitoyl derivative of the inhibitor is 100-fold more effective than the free form supports our previous finding that the enzyme is bound to cellular membranes. We have also reported that the protease is calcium-dependent and acts at neutral pH (Klenk et al., 1984). Cleavage of the FPV hemagglutinin is a late event in intracellular transport and is assumed to occur shortly before the glycoprotein arrives at the plasma membrane (Klenk et al., 1981). The enzyme responsible for activation of the FPV hemagglutinin shows a number of striking similarities in subcellular location and catalytic properties to enzymes believed to process prohormones at pairs of basic residues in endocrine tissues. Proteases cleaving the insulin precursor, that have been isolated from secretory granula of pancreatic β cells, have been found to be under the control of intracanellial calcium and pH (Davidson et al., 1987, 1988). A protease has been isolated from secretory vesicles of the bovine pituitary neural lobe that cleaves proteins and peptides at neutral pH (Parish et al., 1986). The α-factor mating pheromone, a peptide hormone from yeast, is activated at pairs of basic residues by a membrane-bound serine protease that is strictly calcium-dependent and has a neutral pH optimum (Thomas et al., 1988), exactly as has been observed for the activating enzyme of the FPV hemagglutinin. It is therefore reasonable to assume, but remains to be proven, that the enzymes activating viral glycoproteins and peptide hormones belong to the same families of proteinases. Our data show that the enzymes cleave peptidyl-p-nitroanilides with a similar substrate specificity as influenza virus hemagglutinin. Thus,
these chromogenes may substitute for the hemagglutinin, when large amounts of substrate are required, as may be the case, for instance, in studies aimed at the characterization of the activating proteinases.

As has been pointed out above, a large number of viral glycoproteins are cleaved by cellular proteases at arginine–arginine and lysine–arginine sites. It will be interesting to see whether cleavage of these glycoproteins can also be inhibited by the compounds analyzed here. With many of these viruses, e.g., the togaviruses, the coronaviruses, the arenaviruses, the herpes viruses, and most of the retroviruses, the biological significance of the cleavage reaction is not understood. Studies with inhibitors of the type analyzed here should throw light on this problem. In cases, where cleavage of a protein at an arginine-containing site has been suspected, but not definitely proven, the use of peptidyl chloroalkyl ketones might result in the accumulation of polypeptides which so far have not been identified as precursors. The inhibitors might, thus, also help to elucidate new precursor–product relationships.

The list of viruses that depend on proteolytic activation of a glycoprotein to spread in the organism includes HIV 1 (McCune et al., 1988) and many other important pathogens. The application of peptidyl chloroalkyl ketones as antiviral agents is therefore worth considering. As has already been pointed out above, the available evidence indicates that cleavage activation at arginine residues is a mechanism by which also essential cellular functions are regulated. Peptidyl chloroalkyl ketones are therefore usually considered toxic and their application for therapeutic purposes may not be feasible. However, we have shown here that activation of the FPV hemagglutinin is blocked by FAKR-CMK, but not by FAK-CMK. Thus, our data provide evidence for some sequence-specificity. As has been pointed out above, with most viral and cellular proteins the cleavage site consists not only of a pair but of a whole series of basic residues, which all may contribute to its specificity as a recognition signal for the activating proteinases. Furthermore, among different proteins there is considerable sequence variation in the connecting peptides. It may therefore be desirable to construct chloroalkyl ketones with an extended peptide showing exact homology to a specific cleavage site. Such inhibitors may be more selective.

Fig. 3. Multiple cylo replication of FPV in cell culture in the presence of hydrophilic and amphophilic peptidyl chloroalkyl ketones. Cultures of chick embryo cells were inoculated with the Rostock strain of FPV at an m.o.i. of 10^3 PFU per cell and incubated in REM at 37°C. Left: AKR-CMK was added to the medium at concentrations of 0 (○), 1 (△), and 10 mM (■). Right: pal-FAKR-CMK was added at concentrations of 0 (○), 5 (△), 50 (□), and 100 μM (□). At the times indicated, virus released into the medium was measured by hemagglutination titration.
among the proteases they interact with and, thus, be less toxic.

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