The EMBO Journal vol.9 no.12 pp.4189-4195, 1990

An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo

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Communicated by R.Rott

Host cell proteases responsible for activation of viral fusion glycoproteins are an important determinant for spread and tropism of various animal viruses. Exemplifying such proteases for the first time, we isolated an endoprotease from chick embryo, that activates para- and orthomyxovirus fusion glycoproteins by cleaving their precursor proteins at a specific, single arginine site. The protease is a calcium dependent serine protease consisting of two subunits, the 33 kd catalytic chain and the 23 kd chain possibly required for Ca^{2+} binding, and was found to be highly homologous, if not identical, to the blood clotting factor X(FX), a member of the prothrombin family. Its high efficiency and specificity in cleavage reactions was attributable to the properties characteristic of FX. Its role *in vivo* was strongly supported by cleavage inhibition *in ovo* highly selective for this virus group with a specific peptide inhibitor against FX.

Key words: Factor X/fusion glycoprotein/orthomyxovirus/paramyxovirus/proteolytic activation/tissue tropism

Introduction

Viral spreading and tropism in a susceptible host are most often determined by specific receptor, as best exemplified by the proteinaceous receptors for human immunodeficiency virus and picornaviruses (for a review see White and Littman, 1989). Receptors for several viruses, however, are sialic acids on host cell glycoproteins and glycolipids. They are ubiquitous so that the viral tropism cannot be explained by virus-receptor interactions. Instead, the determinants are the viral fusion glycoproteins and the cellular proteases required for their activation. Typical examples for this are paramyxoviruses such as Newcastle disease virus (NDV) in birds and Sendai virus in mice. The paramyxovirus fusion (F) glycoprotein induces fusion between the viral envelope and host cell membrane, thereby enabling entry of the viral genome into host cells. The fusion activity is expressed by post-translational proteolytic cleavage by host cell proteases at an arginine-containing site of the inactive precursor glycoprotein F_0 (Homma and Ohuchi, 1973; Scheid and Choppin, 1974). The F_0 of virulent NDV strains is activated by a ubiquitous protease(s) and the infection is consequently pantropic or systemic, while avirulent strains undergo F_0 cleavage only in a few limited tissue types, hence causing an infection localized to particular organs such as respiratory and alimentary tracts (Nagai *et al.*, 1976a, 1979, 1989). Sendai virus also causes a localized infection. The presence of a specific protease(s) in mouse lung is essential for the F_0 cleavage and viral spreading to cause pneumonia (Tashiro and Homma, 1983a,b). No remarkable virus growth in the other organs appeared to be due to the absence of the activating protease. Exactly the same mechanism is responsible for infection of influenza A viruses of orthomyxoviridae, in which the cleavability of haemagglutinin (HA) determines whether the virus targets a wide spectrum of tissues or shows a narrow tissue range (Bosch *et al.*, 1979; Klenk and Rott, 1988; Webster and Rott, 1987). The amino acid sequence at the cleavage site appears to be the most important determinant for the different cleavability of the glycoproteins. The readily cleavable glycoproteins usually have two or more basic residues at this position whereas a single arginine is found with those of limited cleavability (Bosch *et al.*, 1981; Glickman *et al.*, 1988; Toyoda *et al.*, 1987, 1989). To date, however, little is known about the proteases involved in the cleavage. Identification and characterization of the proteases are an immediate need for further understanding of viral pathogenesis and are also important from the viewpoint that the proteases may serve as the target of antiviral strategy.

Here, we have attempted to isolate a virus activating protease (VAP) that cleaves the precursor glycoproteins of Sendai virus, avirulent NDV and human influenza virus A at a single arginine site. The problem in using homogenate of solid tissues such as mouse lung as the enzyme source would be that it contains various proteases of intracellular and vascular origin, some of which might activate these viruses *in vitro* without necessarily being natural activators. A much simpler experimental host, chick embryo (CE), also clearly realizes protease dependent tropism of these viruses, since efficient virus spreading in the allantoic sac as well as in the amniotic sac is due to the presence of a trypsin-like VAP in the fluids filling these sacs (Muramatsu and Homma, 1980; Appleyard and Davis, 1983). The allantoic fluid (ALF) is available in large amounts without much contamination with blood and tissue, and was used here as the enzyme source. An attempt to isolate a VAP from ALF was made but was unsuccessful; the instability of the enzyme was held to be reponsible for the loss of activity during isolation (Muramatsu and Homma, 1980). We found, however, that the loss of activity was not due to instability but was a result of its strong tendency to cosediment with calcium salts. This property proved to be rather crucial for successful isolation.

Results

Isolation of VAP from ALF

Table I summarizes the steps in VAP isolation with details given in Materials and methods. Activation of Sendai virus

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Table I. Purification of VAP from ALF

Step	Volume (ml)	Total protein (mg)	Yield (%)	Relative specific activity
1. ALF	3500	266	(100)	(1)
2. 10 K Pelleting	300	22.2	77	9
3. Hydroxyapatite	55	0.77	28	98
4. Mono Q FPLC	2	0.110	17	406
5. Sephacryl S-200 HR and Mono Q FPLC	1	0.004	5	3230

hemolysis was the measure of enzyme activity throughout. The starting material was ~3.5 l of ALF (step 1). The next step of pelleting by centrifugation at ~10 000 *g* for 10 min (= 10 K Pelleting, step 2) was initially employed to clarify the starting material, with the enzyme activity being expected to remain in the supernatant. However, the bulk of the activity was found to be pelleted, but was then readily recovered in a soluble form by treating the pellet with EDTA and carrying out dialysis. The pellet had arisen from the cosedimentation of VAP with calcium salts readily formed after thawing the ALF stored at −80°C. The material was then adsorbed to and eluted from hydroxyapatite (step 3) and subjected to Mono Q FLPC (step 4). The Mono Q FLPC yielded a sharp peak of activity at the fractions around 0.4 M NaCl, which was enriched with VAP ~400 times relative to the starting material (Table I), but was found to contain some 10 different proteins species by SDS−PAGE (data not shown). Final purification was accomplished by either gel filtration (Figure 1A) followed by concentration with Mono Q FPLC (step 5), which increased specific activity ~3000 times (Table I) or by PAGE in the absence of both SDS and 2-mercaptoethanol (2 ME) (Figure 1B). The VAP isolated was a 55 kd heterodimer consisting of a 33 kd and a 23 kd subunit (Figure 1C). Selective labeling of the 33 kd subunit with [^{3}H]diisopropylfluorophosphate (DFP) (Figure 1C) confirms the foregoing suggestion that VAP is a serine protease (Muramatsu and Homma, 1980) and demonstrates that this subunit is the catalytic chain.

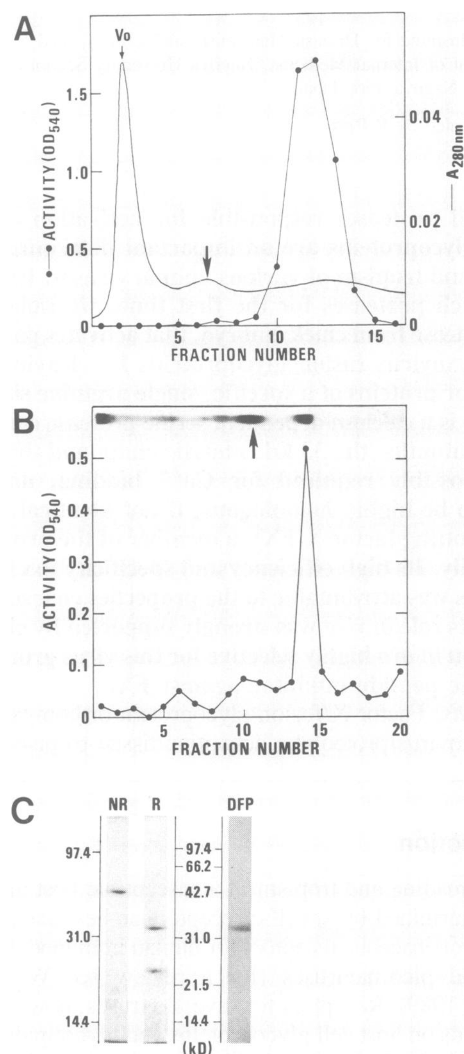

Fig. 1. Chromatography by Sephacryl S-200 HR (**A**) and non-denaturing PAGE (**B**) of the VAP-containing material obtained by Mono Q chromatography of step 4 in Table I. SDS−PAGE (**C**) under non-reducing (NR) and reducing (R) conditions of the VAP from fraction 14 in B. Gels were stained with Coomassie blue. The fluorography of SDS−PAGE of [^{3}H]DFP labeled VAP is also shown (C). Bold arrows in A and B indicate the positions of the precursor of VAP (see text). V_0, void volume.

Formation of VAP from a biologically inactive precursor by proteolytic cleavage

Mono Q chromatography (step 4) always copurified VAP and another heterodimeric molecule of 75 kd, whose smaller subunit comigrated exactly with the 23 kd subunit of VAP on SDS−PAGE, and whose larger subunit was 52 kd. The heterodimer was purified from the peak marked by an arrow in Figure 1A or B, and treated with Russell's viper venom (RVV) which is known to proteolytically activate certain zymogens. The treatment converted the 75 kd polypeptide to a 55 kd heterodimer consisting of a 33 kd and a 23 kd subunit, with a removal of an 18 kd fragment and with a dramatic increase in VAP activity (Figure 2). The N-terminal amino acid sequences of both the 33 kd and the 23 kd chain produced by RVV were identical to those of the naturally occurring VAP shown below. These results demonstrate that the 75 kd molecule is the inactive precursor (pVAP) for VAP.

Characterization of VAP and its specificity in cleavage activation

As already pointed out, VAP is a serine protease whose active site is in the 33 kd chain. We further found that VAP is inhibited by EDTA and EGTA (data not shown), is dependent on calcium ions, showing a sigmoidal Ca^{2+} titration curve with a relatively high Ca^{2+} concentration (>1.2 mM) for maximum activity (Figure 3A), and possesses a neutral pH optimum (Figure 3B). The VAP cleaved the F_0 of Sendai virus and of an avirulent NDV strain Ulster and the HA of a human influenza virus into

the respective subunits, F_1 and F_2, or HA_1 and HA_2 (Figure 4). The F_2 comigrated with the bromophenol blue dye and was not resolved under the SDS−PAGE conditions employed. The F_1 of NDV comigrated with phosphoprotein (P), and was identified by using $[^3H]$glucosamine labeled virions (data not shown) as described (Nagai *et al.*, 1976b). The VAP treatment resulted in a dramatic enhancement of hemolytic activity and infectivity of the paramyxoviruses (Table II). The influenza virus was already somewhat infectious before VAP treatment, even though the hemolysis activation was quite distinct. The reason for this is unknown. The paramyxovirus hemagglutinin-neuraminidase (HN) protein is anchored to the lipid bilayer by its N-terminal region (Blumberg *et al.*, 1985b). Being the precursor for HN, the HN_0 has been found in the least virulent NDV strains, and is likewise proteolytically activated *in ovo* through the removal of a small fragment from the C-terminus (Nagai and Klenk, 1977; Schuy *et al.*, 1984). This cleavage was also induced by the VAP (Figure 4) with an increase in hemagglutinating activity of the virus (Table II). The VAP could neither cleave nor activate the F_0 of Sendai virus TR mutant (Figure 4, Table II), which has isoleucine instead of arginine at the cleavage site and is no longer activated *in ovo* or by trypsin *in vitro* (Itoh *et al.*, 1987). These results indicate that VAP can specifically and correctly process the viral precursors.

Of a number of proteases, trypsin has most often been used for cleavage activation studies *in vitro*, because of its high effectiveness. VAP, however, exhibited features of activation that have not been seen with trypsin. Firstly, full activation of Sendai virus hemolysis required only 0.06 μg/ml of VAP (Figure 3C), which compares with 0.5 μg/ml for trypsin in bringing on nearly full activation. Since it is about twice the size of trypsin, VAP was some 20 times more effective than trypsin on a molar basis. Secondly, although trypsin maintained a high hemolytic activity as shown in Figure 3C, it caused extensive non-specific loss of Sendai virus infectivity at concentrations >1.0 μg/ml under incubation conditions similar to those employed here (Appleyard and Davis, 1983). In contrast, the VAP maintains a dramatic difference in infectivity between treated and untreated Sendai virions (Table II). These results indicate that the VAP is much more effective and specific in cleavage reaction than trypsin.

High homology of VAP with the blood clotting factor Xa

The N-terminal amino acid sequences of both the 23 kd and the 33 kd subunit were found to be highly homologous to the prothrombin family of blood clotting enzymes (Figure 5). Homology was particularly high with the human or the bovine FX, more strictly with each subunit of its activated form FXa. An initial attempt at gas phase sequencing by a standard protocol failed to identify the glutamic acid residues at positions 6, 7 and 14 in the 23 kd chain, which are conserved in the prothrombin family. These three and an additional seven to nine glutamic acids in each N-terminus of the prothrombin family's clotting proteins are known to be post-translationally modified to γ-carboxyglutamic acid (Gla) in a vitamin K dependent process and serve as Ca^{2+} binding sites (Furie *et al.*, 1979; Furie and Furie, 1988). Since heating of Gla containing proteins at 100°C for 5 h in dryness decarboxylates the Gla to yield glutamic acid

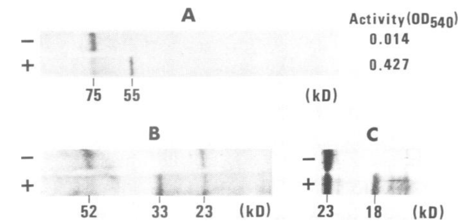

Fig. 2. The precursor, pVAP, isolated from the fractions marked by an arrow in Figure 1A was treated (+) by RVV (6 μg/ml) for 15 min at 37°C or left untreated (−), and analyzed by SDS−PAGE under non-reducing (**A**) and reducing (**B**,**C**) conditions. Gels were silver stained. The 18 kd peptide was detectable only after a prolonged exposure of the gel to the developing solution (C). The capacity to activate Sendai virus by pVAP and VAP is expressed by the viral hemolysis (OD_{540}) (A).

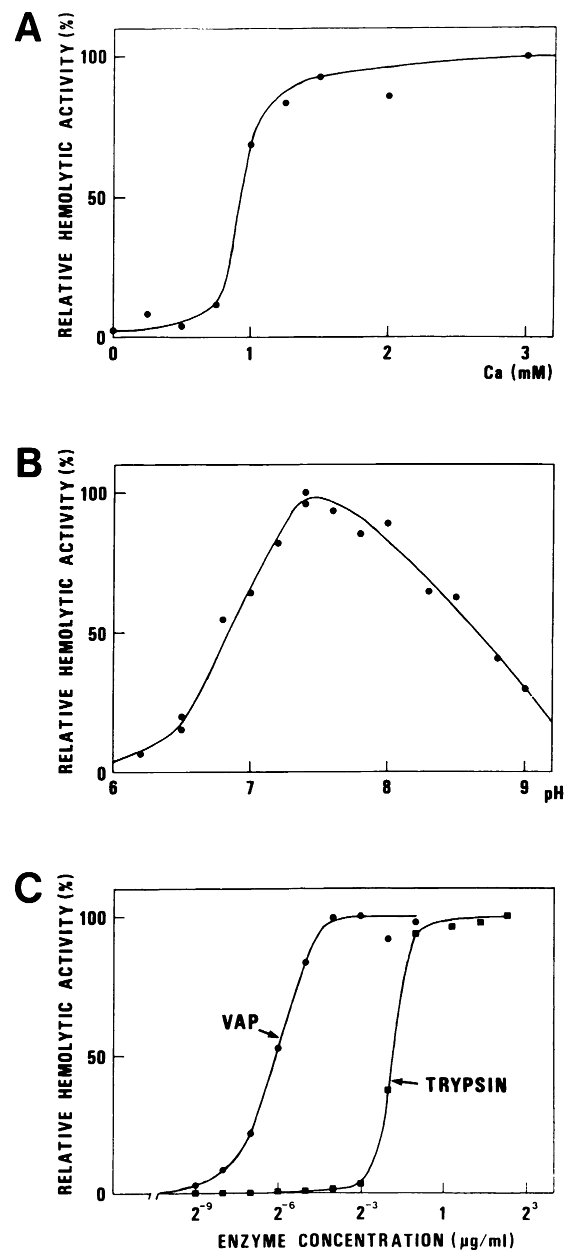

Fig. 3. Effect of Ca^{2+} (**A**) and pH (**B**) on VAP activity and a comparison of virus activation by VAP and trypsin, to which the virions were exposed for 90 min at 37°C (**C**). Activity is expressed as relative hemolytic activity of Sendai virus.

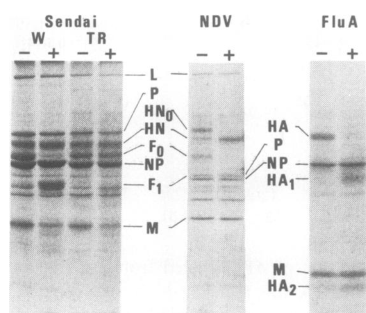

Fig. 4. SDS−PAGE of Sendai virus wild type (W) and TR mutant (see text), NDV Ulster strain and influenza virus A (Flu A) PR8 strain that were treated (+) with 1.0 μg/ml of purified VAP or left untreated (−). Approximately 30 μg of virions were used as the substrate and incubated with or without VAP for 60 min at 37°C. Gels were stained with Coomassie blue.

Table II. Enhancement of viral activities by VAP

Virus	VAP	Hemolysis (OD_{540})	HAU	Virus yield (HAU)
Sendai	−	0.040	2^5	<2
	+	1.637	2^5	2^6
Sendai(TR)	−	0.023	2^4	<2
	+	0.020	2^4	<2
NDV	−	0.022	2^4	<2
	+	0.558	2^7	2^6
Influenza A	−	0.055	2^8	2^4
	+	1.839	2^8	2^7

The virions were treated (+) with VAP (1 μg/ml) for 90 min at 37°C or left untreated (−), and assayed for hemolysis and HAU. The treated and untreated virions were also examined for their infectivity by measuring the HAU produced after a single cycle growth as described in Materials and methods.

without affecting other amino acid residues as well as peptide bonds (Poser and Price, 1979), we heated the 23 kd blot membrane prior to sequencing and could thereby identify the three Glu residues. Thus, these Glu residues are most likely γ-carboxylated and constitute the Gla domain essential for Ca^{2+} binding. Remarkably, the 33 kd subunit begins with the sequence Ile-Val-Gly-Gly, which is characteristic of the catalytically active serine protease chains and marks the point at which the precursor is cleaved to produce an active protease. This N-terminal sequence further shares homology with the N-terminus of trypsin or chymotrypsin, representing one of the conserved regions in the trypsin superfamily of serine proteases (Furie *et al.*, 1982). The unidentifiable amino acids at positions 7 and 12 could be cysteine, since this residue cannot be identified by the sequencing method employed and is present at the corresponding positions in human and bovine FXa (Figure 5). The homology of VAP to FX was finally established by direct sequence comparison in each subunit between the VAP and chicken FXa isolated here (Figure 5). Besides, the FXa was formed by RVV from the FX with essentially the same processing pattern as found for the generation of VAP from pVAP (data not shown). The molecular masses of the precursor and the processing products were identical between the clotting factor and the virus activating protease except that the heavy chain of FXa (35 kd) was slightly larger than that of VAP (33 kd).

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LIGHT CHAIN

        1        10
VAP     ANSFLEEMKQGNIER
HFX     ---------K-HL--
BFX     -------V----L--
HPT     --T----VRK--L--
HFVII   --A----LRP-SL--
CFX     ---------------


HEAVY CHAIN

        1        10        20
VAP     IVGGDEXRPGEXPXQAVLINE
HFX     ----Q-CKD--C-W--L----
BFX     ----RDCAE--C-W--L-V--
HPT     --E-SDAEI-MS-W-VM-FRK
HFVII   ----KVCPK--C-W-VL-LVN
BTR     ----YTCGANTV-Y-VS-NSG
BCH     --N-E-AV--SW-W-VS-QDK
CFX     ------------------X-
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Fig. 5. Alignment of N-terminal amino acid sequences of the 23 kd light and the 33 kd heavy chain of VAP with human factor X (HFX), bovine factor X (BFX), human prothrombin (HPT), human factor VII (HFVII), bovine trypsin (BTR), bovine chymotrypsin (BCT) and chicken factor X (CFX). The single letter code is used. The shadowed E residues in VAP and CFX were identified only after heating at 100°C and those in the other clotting factors are γ-carboxylated (see text). X, unidentified.

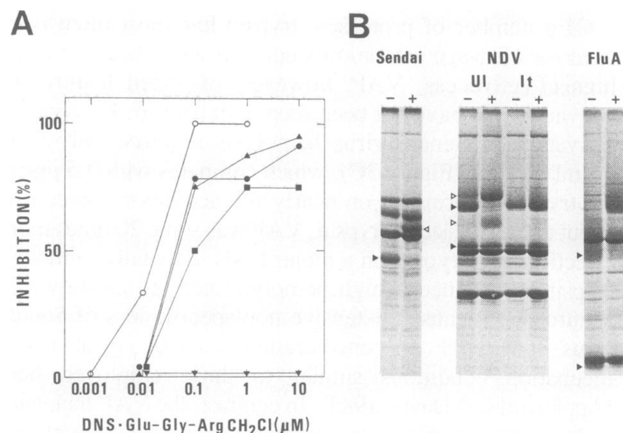

Fig. 6. (**A**) Effect of DNS·Glu-Gly-ArgCH_2Cl on Sendai virus activation by VAP *in vitro* (○) and on the growth in CAM of Sendai virus (●), NDV Ulster(U1) strain (▲), Italien (It) strain (▼) and influenza virus PR 8 (■). (**B**) The effect of the peptide inhibitor on the glycoprotein cleavage of these viruses *in ovo*. ▷, precurors (F_0, HN_0 or HA); ▶, cleavage products (F_1, HN or HA_1 and HA_2). Eggs were treated with the inhibitor (+) by intra-allantoic injection or left untreated (−).

Involvement of VAP in cleavage activation in vivo

FXa participates in a middle phase of the blood clotting cascade by cleaving prothrombin. An important factor determining the strict substrate specificity of FXa is that it cleaves peptide bonds only after a certain arginine. The two hydrolysis sites for FXa in bovine prothrombin are Ile-Glu-Gly-Arg↓ Thr or Ile. By using a series of chloromethylketone peptides whose sequences were deduced from the cleavage site or from unrelated sequences, Kettner and Shaw (1981) revealed that the Glu-Gly-Arg sequence is crucial for the substrate specificity, and a very similar sequence is conserved at the cleavage sites of viral glycoproteins (see Discussion). Moreover, the above authors have developed a highly specific inhibitor, dansylated(DNS)·Glu-Gly-ArgCH_2Cl,

against FXa. We found that this drug strongly inhibited the cleavage activation of Sendai virus by VAP *in vitro* at such low concentrations as 10−100 nM (Figure 6A), now suggesting that the VAP and FXa are not only structurally but also functionally similar to each other. This prompted us to use the inhibitor for assessing the role of VAP *in ovo*. However, a small peptide of this size is, in general, rapidly absorbed and metabolized *in vivo*, making its use unfeasible, in particular, for a long term experiment such as virus spreading by multiple rounds of replication. We therefore examined whether the drug could inhibit glycoprotein cleavage during a single cycle replication. An intra-allantoic injection (0.1 ml, 10 mM) of the drug was made simultaneously with virus inoculation ($\sim 10^8$ p.f.u.) by the same route, and an additional shot of the same dose was given 9 h post infection (p.i.). The virions were collected from the ALF 18 h p.i. and examined for their glycoprotein composition by SDS−PAGE. The inhibitor clearly suppressed cleavage for all three viruses, precursors (F_0, HN_0 or HA) remaining unprocessed in the virions (Figure 6B). The specificity and selectivity of this inhibition was strongly supported by absolute lack of inhibition of F_0 cleavage for a pantropic NDV strain Italien, which has a multiple basic cleavage signal, is activated by a ubiquitously occurring protease, and is therefore used as a control (Figure 6B). With this strain, HN but not HN_0 is the primary translation product (Nagai *et al.*, 1976a). The allantoic cavity of CE is surrounded with chorioallantoic membrane (CAM) which consists of three different germinal layers, the endoderm, the ectoderm and the mesoderm. The viruses with which we are concerned target only the endoderm constituting the inner surface of the wall of the cavity, and reach a high titer in the ALF. This tissue tropism has been well reproduced in an organ culture of CAM (de-embryonated egg) (Nagai *et al.*, 1979; Muramatsu and Homma, 1980). This was used for a long term experiment to examine the effect of inhibitor on virus spreading by multiple replication cycles. The infection was initiated on the endodermal surface with a small virus dose ($\sim 10^3$ p.f.u.), and final virus yields (HAU) at 48 h p.i. were compared in the presence or absence of the drug added to the maintenance media. As shown in Figure 6A, the drug inhibited all three viruses with a similar potency, while it was again absolutely ineffective against the pantropic NDV Italien strain. These results taken together strongly suggest the implication of VAP in cleavage activation *in vivo*.

Discussion

We isolated from CE an endoprotease, designated VAP, which activates para- and orthomyxovirus fusion glycoproteins by cleaving them at a single arginine site and showed that the VAP is a Ca^{2+} dependent serine protease very similar, if not identical, to FXa. Its role *in vivo* was strongly supported by inhibition of cleavage activation with a highly specific inhibitor against FXa. The importance of VAP as a determinant of virus tropism has been further substantiated by a completely different approach using specific antibody and cDNA probes for VAP, which reveals that the sites of virus spreading and expression of VAP, not of pVAP, strictly correlate with each other in the whole embryo (manuscript in preparation). Compared with trypsin VAP is special in its striking effectiveness and specificity of action. These features could be explained by the properties characteristic of FXa. The proteolytic conversion of prothrombin to thrombin by FXa requires the assembly of prothrombin with a complex, which is known as the prothrombinase complex and comprises FXa, the cofactor Va (FVa) and a lipid bilayer (Mann *et al.*, 1982, 1988). Ca^{2+} bound to the Gla residues in FXa and prothrombin plays a key role in the prothrombinase complex formation and the entry of substrate into the complex. The participating molecules are thereby co-concentrated, resulting in manifold magnitude increase of reaction rate. We assume a similar role of Ca^{2+} in virus activation. Ca^{2+} bound to VAP could facilitate the enzyme access to the viral lipid bilayer already complexed with the substrate, resulting in increase of the local enzyme concentration, hence in efficient cleavage. The sigmoidal curve of Ca^{2+} titration (Figure 3A) is compatible with the concept that Ca^{2+} binding to Gla-containing proteins is cooperative (Bloom and Mann, 1978). However, the virus activation differs from the prothrombin activation in that the latter absolutely requires the cofactor FVa that facilitates the enzyme−substrate interactions possibly by bridging the two molecules (Mann *et al.*, 1988). In the former, some other viral envelope components such as the paramyxovirus HN glycoprotein may substitute for the cofactor, or the enzyme−substrate interactions may be strong enough in the absence of the cofactor.

As pointed out above, the Glu-Gly-Arg sequence in prothrombin is important for the strict substrate specificity of FXa. Furthermore, the antepenultimate Glu appeared to be much more important than the following Gly, but could be replaced with glutamine, suggesting that the carbonyl side chain but not the negative charge is required for the binding (Kettner and Shaw, 1981). Interestingly, the viral substrates studied here possess very similar sequences at the cleavage sites (Blumberg *et al.*, 1985a; Toyoda *et al.*, 1987; Winter *et al.*, 1981), that can be generalized as Gln(or Glu)-X-Arg, where X is Thr, Ser or Gly. Moreover, it became evident from the GenBank database that all but one of the 39 influenza virus HAs with a single arginine cleavage site possess this consensus sequence. The cleavage site, Glu-Ala-Arg of NDV HN_0 (Gorman *et al.*, 1988) is also compatible with the consensus signal. Thus, the antepenultimate Glu or Gln could also be important for the specific virus activation *in ovo*. The cleavage site sequence of F_0 was identical between the egg adapted Sendai virus used here and a wild type Sendai virus, which had been serially passaged in mouse lung and was highly pathogenic for mice (unpublished). Similarly, the cleavage site sequences of HA were identical before and after egg adaptation of influenza A viruses that had been isolated in MDCK cells by using trypsin with a broad specificity as an activator (Robertson *et al.*, 1987). These data suggest that FXa-like proteases could also be a very efficient activator for these viruses in higher organisms. Possible activators of this kind are, besides FXa, tryptase in mast cells and urokinase involved in fibrinolysis, since they exhibit a substrate specificity similar to that of FXa (Kettner and Shaw, 1981; Kido *et al.*, 1985). However, either of them failed to activate Sendai virus F_0 protein *in vitro* (T.Yoshida, personal communication). Another candidate is plasmin with a trypsin-like broad specificity. Indeed, some influenza A strains were activated by plasmin, but others and Sendai virus were remarkably insensitive (Appleyard and Davis, 1983; Tashiro *et al.*, 1987). In higher

animals, the FX and the other vitamin K dependent clotting enzymes are, in most instances, synthesized in liver and circulate in body as plasma proteins. They are present in an inactive precursor form until the blood clotting cascade reaction takes place. Therefore, it is unexpected that an FXa-like, active protease is present in the extravascular fluids. Physiological roles of VAP are also an interesting matter to be explored, in view of a number of diverse activities, besides blood clotting, exhibited by the enzymes in the prothrombin family (Shuman, 1986).

Materials and methods

Assay of VAP activity

As the substrate, we used purified Sendai virions that had been grown in $LLCMK_2$ cells and contained exclusively inactive F_0 precursor protein. One hundred μl of the virus [3200 hemagglutinating units (HAU)] in 20 mM Tris–HCl pH 7.4, 150 mM NaCl (TN) was mixed with 100 μl of serial 2-fold dilutions with TN of the fractions to be tested and was then incubated at 37°C for 1 h in the presence of 2.5 mM $CaCl_2$. After adding 50 μl of 0.01% soybean trypsin inhibitor and 100 μl of 50 mM EDTA the reaction mixture was mixed with 1 ml of 2% chicken erythrocytes in phosphate buffered saline (PBS), and was further incubated at 37°C for 1 h. The released hemoglobin was measured by absorbance at 540 nm (OD_{540}). The VAP activity was expressed by OD_{540} within a range where the amount of VAP and hemolysis was proportional. Activation of NDV (strain Ulster) and influenza A virus (PR/8/34) was performed similarly except that the influenza virus was grown in BHK cells and that its hemolysis was measured at a low pH by resuspending the erythrocytes bound with the virions into 0.1 M sodium acetate (pH 5.0) with 0.1 M NaCl (Huang *et al.*, 1981). The VAP activity to enhance viral infectivity was determined by comparing the virus yields (HAU) between VAP treated and untreated viruses under single cycle growth conditions (Klenk *et al.*, 1975).

Purification of VAP

All the following steps were carried out at 4°C. The ALF (3.5 l) obtained from several hundred 11 day old CE was stored at −80°C. The thawed fluid was centrifuged at ~10 000 *g* for 10 min to pellet down the VAP. The pellet was dissolved by 200 ml TN containing 10 mM EDTA and dialyzed against TN for 2 days. The dialysate (300 ml) was adsorbed to 2.0 g hydroxyapatite (Wako, Osaka) and step-wise eluted by each 50 ml of 0.05, 0.1, 0.2 and 0.4 M sodium phosphate buffer (PB), pH 8.0. The eluate by 0.1 M PB containing the bulk of VAP was dialyzed overnight against TN. After centrifugation for 10 min at ~10 000 *g* to remove any insoluble substance, the material was loaded onto a 1 ml Mono Q column pre-equilibrated with TN at a flow rate of 0.5 ml/min. After a 10 ml wash with 0.2 M NaCl in 20 mM Tris–HCl, pH 7.4, VAP activity was eluted into ~0.4 M fractions with a 25 ml linear gradient of 0.2–0.7 M NaCl in the same buffer. Finally, the enzyme-containing fractions were pooled, loaded onto a Sephacryl S-200 HR column (1.5 × 68 cm) and fractionated by TN. The peak fractions collected were condensed by Mono Q FPLC. The final purification was also done by a non-denaturing PAGE of 3.5% stacking and 7.5% separating cylindrical gels without both SDS and 2ME in a buffer system by Laemmli (1970). The gels were sliced into 3 mm fractions, which were then immersed into 100 μl of 0.1 M Tris–HCl pH 7.4 for elution of VAP.

Isolation of FX from chicken plasma

FX containing crude material was obtained as a Gla-containing fraction from the citrated chicken plasma that underwent precipitation with 0.1 M $BaCl_2$ according to Walz *et al.* (1974). The detection of FX in all purification steps was undertaken with a specific assay kit containing RVV and a specific artificial substrate, *N*-benzoyl-L-isoleucyl-L-glutamyl-(γ-piperidyl)-glycyl-L-arginine-*p*-nitroanilide HCl (KabiVitrum AB). The Gla-containing fraction was dialyzed against 20 mM citrate buffer pH 7.5 and loaded onto a Q-Sepharose Fast Flow column (1.5 × 13 cm) (Pharmacia, Stockholm) pre-equilibrated with the same buffer. The FX was eluted into ~0.35 M fractions with a linear gradient of 0.02–0.5 M citrate. The pooled fractions were dialyzed against 50 mM imidazole–HCl, pH 6.0 and after adding $CaCl_2$ at 2.5 mM they were further fractionated by a heparin–Sepharose CL-6B column (2 × 5 cm) (Pharmacia, Stockholm). The FX was eluted around 0.35 M NaCl with a linear gradient of 0–0.5 M NaCl in 50 mM imidazole–HCl pH 6.0, 2.5 mM $CaCl_2$. After dialysis against TN this fraction was further chromatographed on a Mono Q column and peak fractions were obtained around 0.35 M NaCl. Final purification into homogeneity was done by gel filtration through a Sephacryl S-200 HR (1.5 × 68 cm) and condensation by Mono Q FPLC.

Miscellaneous

Labeling of VAP with [^{3}H]DFP (774 GBq/mmol) (Amersham, Buckinghamshire) was performed in TN containing 6 MBq/ml of [^{3}H]DFP and 2.5 mM $CaCl_2$. The pVAP and FX were treated with RVV (KabiVitrum AB) (6 μg/ml) in TN in the presence of 2.5 mM $CaCl_2$. Acetyl trypsin (15400 units/mg protein) used for virus activation was purchased from Sigma, St Louis, MO. Molecular weight was determined by Lemmli's SDS–PAGE with 12.5% separating and 3.5% stacking slab gels alternatively in the presence and absence of 2ME. Phosphorylase *b* (97.4 kd), bovine serum albumin (66.2 kd), ovalbumin (42.7 kd), carbonic anhydrase (31.0 kd), soybean trypsin inhibitor (21.5 kd) and lysozyme (14.4 kd) (Bio-Rad, Richmond, CA) were used as molecular standards. Gels were stained either by a silver stain kit (Wako, Osaka) or by Coomassie brilliant blue. Protein concentration was measured by the dye binding method (Bradford, 1976). Sequencing of proteins blotted onto PVDF membranes (Millipore, Bedford, MA) was performed on an Applied Biosystems Model 470A protein sequencer (program 03CPTH) with on-line injection onto a model 120A PTH analyzer with a C_{18} reverse-phase column at the Center for Gene Research, Nagoya University. A sequence homology search was done with the GenBank protein sequence database. DNS·Glu-Gly-ArgCH_2Cl was synthesized as described (Kettner and Shaw, 1981), and provided by K.Inoue, Shionogi Medical Science Research Institute (Osaka). The virus growth in CAM was examined by using de-embryonated eggs, in which the CAM remain attached to the shell and maintained with minimum essential medium (Nagai *et al.*, 1979).

Acknowledgements

We are grateful to S.Iwanaga, H.Saito and S.Yoshida for helpful discussions. We also thank M.Homma and K.Inoue for giving the Sendai virus TR mutant and DNS·Glu-Gly-ArgCH_2Cl, respectively, K.Kobayashi and Y.Kato for amino acid sequence analysis, and K.Wada for homology search. B.G. is a recipient of Fellowships of the Japan Society for the Promotion of Science for Japanese Junior Scientists. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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Received on June 6, 1990; revised on August 20, 1990