Volume 296, number 3, 274–278 FEBS 10613 January 1992

Isolation of factor Xa from chick embryo as the amniotic endoprotease responsible for paramyxovirus activation

Bin Gotoh[a], Fumiko Yamauchi[b], Tomohiko Ogasawara[c] and Yoshiyuki Nagai[b]

[a]*Radioisotope Center Medical Division,* [b]*Research Institute for Disease Mechanism and Control* and [c]*1st Department of Internal Medicine, Nagoya University School of Medicine, Nagoya 466, Japan*

Received 18 November 1991

In chick embryo, certain paramyxoviruses mainly target the chorioallantois and the allantoamnion and show no extensive further spreading in the other organs. This has been explained by the possible presence of an endoprotease activating the viral fusion glycoprotein precursor in the allantoic and the amniotic fluid, and its absence in other places or organs. We previously isolated such an endoprotease from the allantoic fluid and demonstrated its identity with the clotting factor Xa. Exactly the same endoprotease by all the criteria including the N-terminal amino acid sequence was now isolated from the amniotic fluid. Thus, the factor Xa seems to be a major host determinant of the viral tropism in chick embryo.

Virus activating protease; Factor X; Viral tropism; Chick amniotic fluid

1. INTRODUCTION

Enveloped animal viruses usually possess a surface glycoprotein which mediates fusion between the viral envelope and host cell membrane. An essential step in the biosynthesis of the fusion glycoprotein is the post-translational endoproteolytic cleavage of the inactive precursor glycoprotein by host cell proteases. For example, the fusion (F) glycoprotein of Sendai virus and Newcastle disease virus (NDV) in paramyxoviridae is synthesized in a form of precursor F0 protein, and the specific endoproteolysis of F0 into two subunits, the N-terminal F2 and the C-terminal F1, is essential for the expression of the viral fusion activity and infectivity [1–3]. The hydrophobic stretch of some 25 amino acids generated at the F1 N terminus by cleavage is thought to be the direct mediator of fusion activity [4]. Depending upon whether the cleavage site consists of a single arginine or di- or oligo-basic residues, the viral F0 protein is activated by a particular protease(s) produced by a few particular tissues or by a ubiquitous protease(s) present in a wide variety of tissues [5–7]. Since the specific receptors for these viruses are widespread sialic acid-containing molecules, the different protease dependency specified by those cleavage motifs in the F0 precursor could be a direct determinant of the viral tropism. In cells cultured in vitro, viruses of the former type require exogenously added trypsin or a trypsin-like enzyme to undergo multiple rounds of replication, while the latter shows no such requirement.

The protease-dependency of viral tropism was strikingly illustrated in studies of virulent and avirulent strains of NDV in chick embryo (CE), where the virulent strains with an oligo-basic cleavage motif spread throughout the embryo, while the infection of avirulent strains with a single arginine motif is limited to certain tissues such as chorioallantois [8]. This simple model host organism further provided another striking example by the use of Sendai virus and its protease-activation mutants [9,10]. The F0 of wild type has a single arginine motif and like avirulent NDV, replicates well in the chorioallantois but does not spread extensively in other organs. The mutants, which were altered to require chymotrypsin or elastase for F0 activation but could no longer be activated by trypsin, now failed to replicate even in the CA. These data suggested that a trypsin-like protease(s) was responsible for Sendai virus activation in the chorioallantois of CE. Furthermore, one of the protease-activation mutants was found to have lost the capacity to produce pneumonea in mice, suggesting the involvement of similar proteases in viral spread and pathogenesis in the natural host [10]. However, no further extensive characterization of the proteases has been done. This is a major obstacle for full understanding of protease-dependent viral tropism. Exemplifing such proteases for the first time, we recently isolated a virus-activating protease (VAP) from CE. This enzyme purified from the allantoic fluid (ALF) filling the CA, specifically cleaved the F0 of Sendai virus and avirulent NDV, and was demonstrated to be identical with the clotting factor Xa (FXa) [11,12]. The amnion sac in CE

Abbreviations: FX, factor X; FXa, activated form of FX; VAP, virus activating protease; CE, chick embryo; AMF, amniotic fluid; ALF, allantoic fluid.

Correspondence address: Y. Nagai, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya 466, Japan. Fax: (81) (52) 731 5844.

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is another major site targeted by these viruses. Is the same protease involved in virus-activation at this site? If different, how diverse can the enzymes be, which participate in activation in different places of a single host organism? To solve these questions, we have here attempted to isolate the responsible amniotic protease and demonstrated that the isolated enzyme is also the FXa, expanding the significance of FXa as a host determinant of viral tropism in CE.

2. MATERIALS AND METHODS

2.1. *Assay of VAP activity*

Sendai virus, grown in LLCMK2 cells and containing exclusively the inactive F0 precursor, was used as the substrate of VAP assay. The VAP assay consists of cleavage of the substrate virion F0 protein followed by measurement of hemolysis, an event resulting from fusion between the viral envelope and the erythrocyte membrane. The reaction mixture consisting of 100 μl of the virus (~3,200 hemagglutinating units) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TN), and 100 μl of serial two-fold dilutions (with TN) of the fractions to be tested, were 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 received 1 ml of 2% chicken erythrocytes in phosphate-buffered saline and was further incubated at 37°C for 1 h. After low-speed centrifugation, the hemoglobin released into the supernatant was measured by absorbance at 540 nm (OD_{540}) [11]. The VAP activities in Table I and the figures were expressed as a relative hemolytic activity within a range where the amount of VAP and hemolysis were proportional.

2.2. *Isolation of VAP from the amniotic fluid*

As in the case of VAP-isolation from the ALF, we used here a fluid filling the target organ, namely the amniotic fluid (AMF). All the steps were carried out at 4°C. The AMF (1.7 liters) was mixed with 170 ml of 3.8% sodium citrate, and was then subjected to precipitation by adding 170 ml of 1 M $BaCl_2$ and centrifugation at 10,000 × *g* for 10 min. The barium citrate precipitates were washed 3 times with 100 ml of distilled water, solubilized by adding 60 ml of 0.5 M EDTA pH 8.0, and dialyzed against 5 liters of 50 mM imidazole-HCl pH 6.0, (buffer IB). After removing insoluble substances by centrifugation at 10,000 × *g* for 10 min, the dialysate was loaded onto a Heparin-Sepharose CL-6B column (2 × 5 cm) (Pharmacia, Stockholm) pre-equilibrated with IB at a flow rate of 0.5 ml/min, and stepwise-eluted with 0.2, 0.6, and 1.0 M NaCl in the same buffer. The 0.6 M NaCl fraction, containing the bulk of VAP, was dialysed overnight against TN and centrifuged at 10,000 × *g* for 10 min, and 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, the elution was performed with a 25 ml linear gradient of 0.2–0.7 M NaCl in the same buffer and the activity was recovered in ~ 0.4 M fractions. The VAP was finally purified into homogeneity by Sephacryl S-200 HR (1.5 × 77 cm) gel filtration.

2.3. *Miscellaneous*

N-terminal amino acid sequencing of proteins blotted onto polyvinilidene difluoride membranes (Millipore, Bedford, MA) was performed as previously described [11] on an Applied Biosystems Model 470 A protein sequencer at the Center for Gene Research, Nagoya University. Sodium dodecyl sulfate polyacryl amide gel electrophoresis (SDS-PAGE) with 3.5% stacking and 10 or 12.5% separating gels was performed according to Laemmli [13]. Gels were Coomassie blue- or silver-stained [11]. Protein concentration was measured by the dye-binding method [14]. Propagation in LLCMK2 cells of Sendai virus, NDV (strain Ulster) and Influenza A virus (strain PR/8/34) and their purification were as previously described [3,11].

3. RESULTS

3.1. *Purification of VAP from the AMF*

Table I summarizes the purification steps of VAP from the AMF (VAPam). The starting material was about 1.7 liters of AMF from uninfected, 11-day-old embryos (Table I, step 1; Fig. 1A, lane 1). The AMF was harvested without any appreciable contamination with ALF [15]. FXa contains γ-carboxyglutamic acid (Gla) residues characteristic of vitamin K-dependent proteins. Here, we took advantage of the features that such proteins are selectively adsorbed onto barium citrate salts [16,17], which had not been taken into consideration for the previous isolation of VAP from the ALF (VAPal). The barium citrate precipitation already resulted in a dramatic enrichment of VAP activity (Table I, step 2). No VAP activity was detected in the supernatant. In the subsequent step of Heparin-Sepharose CL-6B column chromatography, the bulk of VAP was eluted into the 0.6 M NaCl fraction (Table I, step 3). This fraction still contained a number of proteins besides VAP (Fig. 1A, lane 3). The material was then subjected to Mono Q FPLC, which yielded a sharp peak of activity at the fractions 14 and 15 around 0.4 M NaCl (Fig. 1B) with a striking increase in specific activity (Table I, step 4). Final purification was accomplished by Sephacryl S-200 HR gel filtration (Fig. 2A) with about 500-fold increase in the specific activity (Table I, step 5). After concentration of a pool of fractions 13–17 in Fig. 2A by lyophilization, we carried out SDS-PAGE and found that the VAPam was a 55 kDa heterodimer consisting of a 33 kDa and a 23 kDa subunit (Fig. 2B). Thus, both the molecular mass and the subunit composition of the VAPam were identical to those of VAPal [11].

Table I

Purification of VAP from AMF

Step	Volume (ml)	Total protein (mg)	Yield (%)	Relative specific activity
1. AMF	1700	147	(100)	(1)
2. Barium citrate precipitate	100	3.20	72	33
3. Heparin-Sepharose CL-6B	55	1.90	52	40
4. Mono Q FPLC	2	0.162	20	182
5. Sephacryl S-200 HR	10	0.014	5	525

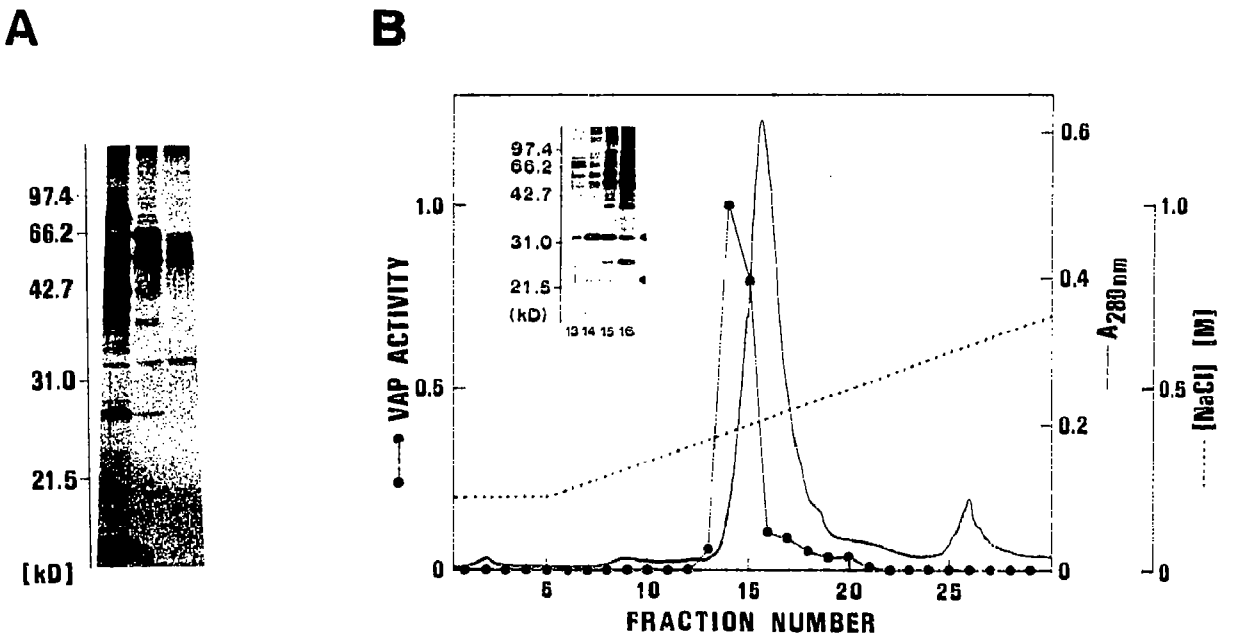

Fig. 1. SDS-PAGE of the VAP-containing materials in each of the purification steps in Table I (A) and the Mono Q chromatography of the materials obtained at step 3 in Table I (B). The numbers on the top of the gel lanes in A correspond to those of the purification steps in Table I. Insert in B, SDS-PAGE of the fractions 13–16. ◀, positions of the heavy and the light chain of VAP. Gels were all silver-stained.

3.2. *Proteolytic activation by VAPam of the viral fusion glycoprotein precursors*

The isolated VAPam could cleave not only Sendai virus F0 but also the F0 of NDV avirulent strain (Ulster) with an enhancement of viral hemolysis (Fig. 3). The cleavage of NDV hemagglutinin-neuraminidase precursor (HNO) into the biologically active hemagglutinin-neuraminidase (HN), was also induced (Fig. 3). Influenza virus hemagglutinin (HA) also requires proteolytic cleavage for expressing membrane fusion activity. The VAPam induced this activation too, as exemplified by PR/8/34 strain of H1 subtype whose HA has a single arginine cleavage-motif and is processed into HA1 and HA2 by the enzyme (Fig. 3). Thus, the VAPam was found to possess a substrate specificity very similar to that of VAPal [11]. The requirement of

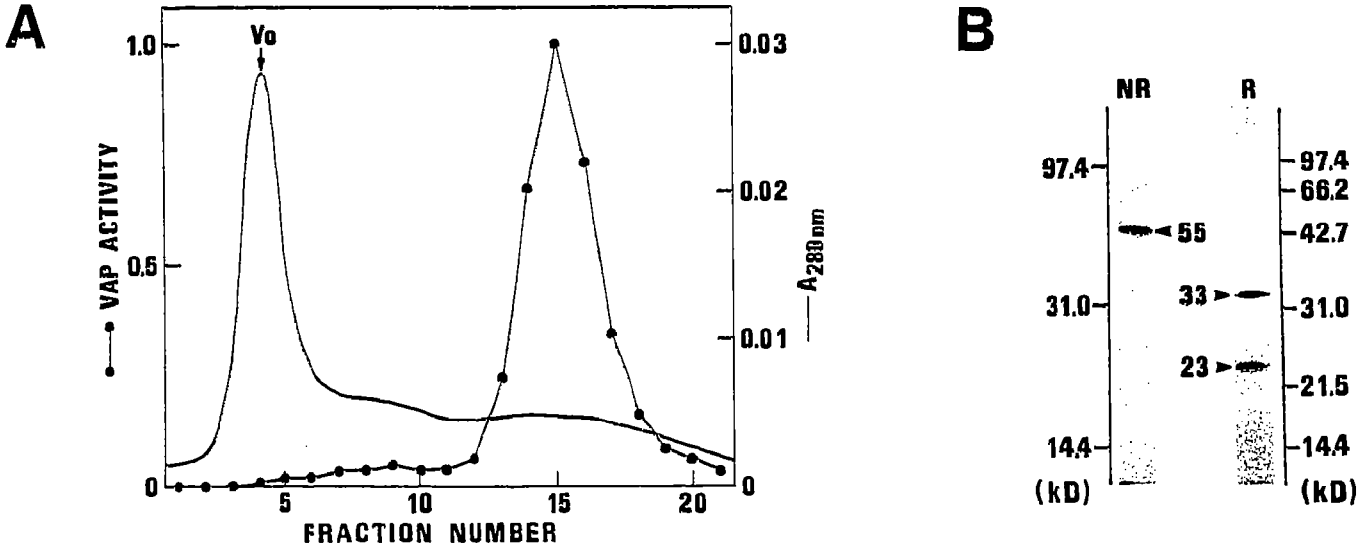

Fig. 2. Chromatography by Sephacryl S-200 HR of the VAP-containing material recovered from the fractions 14 and 15 in Fig. 1B of the Mono Q chromatography (A) and SDS-PAGE under nonreducing (NR) and reducing (R) conditions of the VAP from the pool of fractions (fr. 13–17) in A (B). Vo, void volume.

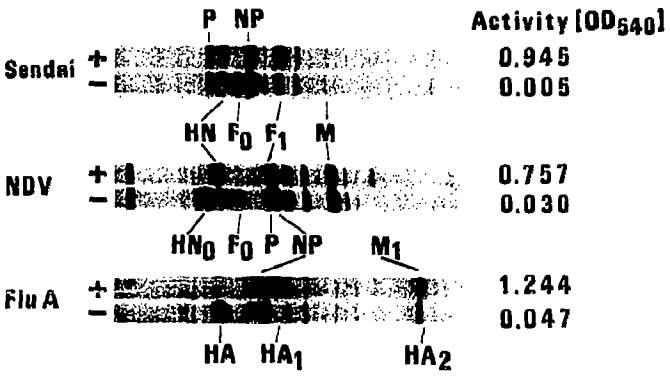

Fig. 3. SDS-PAGE under reducing conditions of Sendai virus, NDV, and influenza virus A (FluA) proteins that were treated (+) with 0.5 μg/ml of purified VAPam for 60 min at 37°C, or left untreated (−). Gels were stained with Coomassie blue, and migration was from left to right. Hemolytic activity of viruses treated or untreated with VAPam is shown on the right of each gel. Hemolysis by the influenza virus was measured at pH 5.0 as described previously [11].

Ca^{2+} (1.2 mM or more) and the pH range (7.2–8.0) for the optimal action of VAPam were also very similar to those found for VAPal [11].

3.3. *N-terminal amino acid sequencing of VAPam*

The identity between the VAPam and VAPal was finally established by amino acid sequencing of the N termini of both the 33 kDa and the 23 kDa chain. As shown in Fig. 4, the sequences were exactly the same amongst the VAPam, VAPal, and the chicken FXa. We should note that Glu residues in the 23 kDa chain of these 3 molecules could only be identified by heating the blotting membranes at 100°C in dryness prior to sequencing, strongly suggesting that these residues are indeed γ-carboxylated to serve as the Ca^{2+} binding sites

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LIGHT CHAIN

          1        10
VAPam     ANSFLEEMKQGNLER
VAPal     ---------------
CFX       ---------------
HFX       ----------K-HL--
BFX       ----------V----L--

HEAVY CHAIN

          1        10        20
VAPam     IVGGDEXRPGEXPWQAVLINE
VAPal     ----------X-------
CFX       ----------X-----X-
HFX       ----Q-CKD--C----L----
BFX       ----RDCAE--C----L-V--
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Fig. 4. Alignment of N-terminal amino acid sequences of the 23 kDa light and the 33 kDa heavy chain of VAPam with those of VAPal, chicken factor X (CFX), human factor X (HFX), and bovine factor X (BFX). Single letter code is used. The shadowed E residues were suggested to be γ-carboxylated [11]. X, unidentified.

[11,18]. The molecular mass of 55 kDa defined here is in good agreement with that of a Sendai virus-activating protease also partially purified from the AMF of CE by Appleyard and Davis [19].

4. DISCUSSION

The results presented here unequivocally show that the virus-activating capacity of AMF is attributable to the FXa present in the fluid, as in the case of the allantoic fluid. In the beginning of this work, however, we did not always expect this conclusion, because there could be a number of different endoproteases in a living organism, or perhaps even in a living cell, which are potentially able to cleave the carboxyl side of an arginine. We previously found the presence of the enzymatically inactive precursor to VAP (pVAP) in the ALF [11]. The molar ratio of pVAP to VAP was about 4-to-1. The pVAP is equivalent to the inactive zymogen, FX, and was converted to VAP by limited proteolysis with Russell's viper venom in the identical manner found for conversion of FX to FXa [11]. In the AMF, however, only the VAP but no detectable pVAP has been found, indicating that the precursor is completely processed there. Thus the metabolism of pVAP appears to be somewhat different in these two fluids, although the native protease(s) for conversion of pVAP to VAP remains to be identified. The concentrations of VAP in the AMF and the ALF were calculated to be approximately 0.16 μg/ml and 0.03 μg/ml, respectively. This difference may explain that Sendai virus activating capacity of the AMF was about 10 times higher than that of the ALF [19].

In order to obtain more direct proof for the in vivo role of FXa in virus activation and spreading, we need a number of studies including the protease gene expression and the processing of the gene products from the precursor to the active form at a tissue level and their correlation with the viral tissue specificity or tropism. These studies have recently demonstrated that although FX is generally synthesized in the liver, many other tissues in the CE, including the chorioallantois and the allantoamnion, produce the FX that is the biologically active FXa, however, present only in the AMF and the ALF, and that the paramyxoviruses replicate only in those tissues in direct contact with these fluids [20]. These results and those presented here and obtained previously [11,12] seem to complement one another very well, and suggest strongly that the FXa is of prime importance as a host determinant of viral tropism in CE. Undoubtedly, the FXa is not present in CE for the sole purpose of activating the viruses. Its normal cellular functions are not known and deserve investigation in view of the facts that prothrombin-like proteases appear to play roles in other biological processes than blood clotting, including cell differentiation and embryogenesis [21–23].

Acknowledgements: We thank A. Sato and S. Sakata, at the Center for Gene Research, Nagoya University, for amino acid sequence analysis, N. Saito for technical assistance, and A. Nakamura and J. Uchida for preparing the manuscript. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Japan.

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