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Primary structure of the virus activating protease from chick embryo

Its identity with the blood clotting factor Xa

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Host cell proteases activating para- and orthomyxovirus fusion glycoprotein precursors play a crucial role in determining the viral tropism in infected organisms. We previously isolated such an endoprotease from the allantoic fluid of chick embryo and showed its close similarity to the activated form of blood clotting factor X (FXa) by partial amino acid sequencing. In this report, we have cloned and sequenced a cDNA of the protease, and show that it is encoded in a single gene as a preproform with all the functional and structural domains known to be characteristic of bovine or human FX, establishing the identity between the protease and FXa.

Virus activating protease; Factor X; Stuart factor; Chick embryo

1. INTRODUCTION

Most, if not all, of enveloped animal viruses possess an envelope glycoprotein, which mediates fusion between the viral envelope and host cell membrane, hence enables the entry of viral genome into cytoplasm. There is now growing evidence that the fusion glycoprotein is activated by posttranslational proteolytic cleavage catalyzed by host cell proteases [1–6]. We recently isolated an endoprotease designated VAP (virus activating protease) from the allantoic fluid of chick embryo [7]. This VAP cleaves the fusion proteins of Sendai virus and Newcastle disease virus in paramyxoviridae and influenza virus A of orthomyxoviridae at a specific single arginine-containing site, and plays a key role in the viral spreading in the allantoic sac. The VAP is a serine protease of 55 kDa heterodimer, consisting of the 23 kDa (light) chain with Ca^{2+} binding capacity and the catalytic 33 kDa (heavy) chain, which are linked to each other by a disulfide bond. The N-terminal amino acid sequence of both chains displayed a striking homology with those of the activated form of bovine FX (BFXa) and human FX (HFXa), a member of vitamin K dependent serine proteases of prothrombin family. The similarity of VAP to the FXa was further substantiated by complete N-terminal sequence match in both chains between the VAP and the chicken (C) FXa isolated from plasma. A highly specific and efficient activation of the viral glycoproteins by VAP was explained by analogy to activation of the natural substrate of FXa, the prothrombin, although no further structural information for either VAP or CFXa was available, including their primary structure [7]. At the same time, however, if the VAP is actually identical with FXa, its presence in the extra-vascular fluid is unexpected, since FX is usually synthesized in liver, and exists in plasma as a biologically inactive form (zymogen) until blood clotting starts. Under these circumstances, there is clearly a need to unequivocally show whether the VAP is identical with FXa or not. Here, we have established the identity by determining the primary structure of VAP, and show that the presence of VAP in the allantoic fluid is a consequence of ectopic expression of the FX gene in the chorioallantoic membrane (CAM).

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Abbreviations: FX, factor X; FXa, activated form of FX; VAP, virus activating protease; CAM, chorioallantoic membrane

2. MATERIALS AND METHODS

2.1. *cDNA cloning of the VAP*

Total RNA was extracted from the CAM of 13-day-old chick embryos by the method of Chomczynski and Sacchi [8], and poly(A)$^+$ RNA was isolated by oligo(dT)-Latex [9]. cDNA was made by using a cDNA synthesis kit (Pharmacia) and cloned into the λgt10. Two sets of oligonucleotide mixture with overlapping sequences, 5′-GARGARATGAARCARGG-3′ and 5′-ATGAARCARGGNAAYAT-3′ (R, Y and N denote A+G, C+T and A+G+C+T, respectively), which were deduced from the N-terminal amino acid sequence of the light chain of VAP, were used for hybridization selection at 41°C and 37°C, respectively. The cDNA inserts of positive

clones were subcloned into pBluescript II (Stratagene) and the nucleotide sequencing was performed by the method of Sanger et al. [10] using deletion mutants prepared by the Exonuclease III [11].

2.2. *RNA blot hybridization analysis*

Poly(A)$^+$ RNA from tissues, prepared as described above, was denatured by glyoxal and electrophoresed on 1% agarose gel [12]. The Hybond-N nylon membrane (Amersham) was used for blotting. Hybridization was performed at 42°C in the presence of 5 × SSPE, 5 × Denhardt, 50% formamide, 0.5% SDS and 20 μg/ml salmon sperm DNA. A 3'-non-coding region of the VAP cDNA (nucleotide residues 1424–2195 in Fig. 1) was used as a specific probe.

3. RESULTS

3.1. *Molecular cloning of VAP*

Because the allantoic fluid from which VAP was isolated consists of liquid derived from the CAM and excretions from kidney and cloaca [13], and because the cultivated CAM cells in vitro still retain a potency to proteolytically activate the viruses [5], we tentatively assumed that VAP would be derived from the CAM and used it as the source of RNA for the preparation of a cDNA library. The library was screened by hybridization with two sets of 17-mer oligonucleotide mixtures as described in section 2. Three positive clones designated pCHP9, pCHP12 and pCHP21 were obtained from approximately 300000 plaques and their nucleotide sequences were determined. Fig. 1 shows the 2246-nucleotide cDNA sequence for VAP. The primary structure of VAP was deduced by using the reading frame corresponding to the previously determined amino acid sequence of VAP (amino acid residues 41–55 and 241–261, underlined in Fig. 1) [7]. The

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-51                                                  5'----CTT CCC GTC CCG TCC CGT CCC GTC CCG TCG CGG TGC CCA TCC CGA GCC ACC    -1

  1 ATG GCC GGC CGC CTG CTG CTC CTG CTG CTC TGC GCG GCG TTG CCG GAC GAG CTC CGG GCT GAA GGA GGC GTG TTC ATC AAG AAA GAA AGT     90
  1 Met Ala Gly Arg Leu Leu Leu Leu Leu Cys Ala Ala Leu Pro Asp Glu Leu Arg Ala Glu Gly Gly Val Phe Ile Lys Lys Glu Ser     30

 91 GCC GAC AAG TTC TTG GAA AGA ACA AAA CGT GCC AAC TCT TTT TTA GAA GAA ATG AAG CAA GGC AAT ATT GAA AGA GAA TGC AAC GAG GAG    180
 31 Ala Asp Lys Phe Leu Glu Arg Thr Lys Arg Ala Asn Ser Phe Leu Glu Glu Met Lys Gln Gly Asn Ile Glu Arg Glu Cys Asn Glu Glu     60

181 CGC TGC TCA AAA GAA GAG GCA AGA GAA GCC TTT GAA GAC AAT GAG AAA ACT GAG GAA TTC TGG AAT ATC TAC GTA GAT GGC GAC CAG TGC    270
 61 Arg Cys Ser Lys Glu Glu Ala Arg Glu Ala Phe Glu Asp Asn Glu Lys Thr Glu Glu Phe Trp Asn Ile Tyr Val Asp Gly Asp Gln Cys     90

271 AGC TCA AAT CCA TGT CAC TAT GGT GGA CAA TGT AAA GAT GGA CTT GGT TCC TAC ACT TGC TCC TGT TTG GAT GGT TAT CAA GGC AAG AAC    360
 91 Ser Ser Asn Pro Cys His Tyr Gly Gly Gln Cys Lys Asp Gly Leu Gly Ser Tyr Thr Cys Ser Cys Leu Asp Gly Tyr Gln Gly Lys Asn    120

361 TGT GAA TTT GTC ATA CCG AAG TAC TGC AAA ATA AAC AAT GGT GAC TGT GAG CAG TTC TGC AGC ATC AAA AAA AGC GTG CAG AAG GAT GTC    450
121 Cys Glu Phe Val Ile Pro Lys Tyr Cys Lys Ile Asn Asn Gly Asp Cys Glu Gln Phe Cys Ser Ile Lys Lys Ser Val Gln Lys Asp Val    150

451 GTG TGT TCC TGT ACA AGT GGG TAT GAG CTG GCA GAA GAT GGC AAA CAG TGT GTT TCA AAA GTA AAG TAC CCA TGT GGA AAA GTT CTC ATG    540
151 Val Cys Ser Cys Thr Ser Gly Tyr Glu Leu Ala Glu Asp Gly Lys Gln Cys Val Ser Lys Val Lys Tyr Pro Cys Gly Lys Val Leu Met    180

541 AAA AGA ATT AAA AGG TCT GTC ATC TTA CCC ACT AAT AGT AAT ACC AAT GCA ACT AGT GAT CAA GAT GTC CCC TCC ACG AAT GGA TCA ATT    630
181 Lys Arg Ile Lys Arg Ser Val Ile Leu Pro Thr Asn Ser Asn Thr Asn Ala Thr Ser Asp Gln Asp Val Pro Ser Thr Asn Gly Ser Ile    210

631 CTG GAG GAG GTC TTT ACT ACT ACC ACA GAA AGC CCA ACT CCC CCT CCT CGC AAC GGA TCG AGT ATC ACA GAT CCA AAT GTC GAT ACC AGG    720
211 Leu Glu Glu Val Phe Thr Thr Thr Thr Glu Ser Pro Thr Pro Pro Pro Arg Asn Gly Ser Ser Ile Thr Asp Pro Asn Val Asp Thr Arg    240

721 ATA GTA GGT GGG GAT GAG TGT CGT CCT GGT GAA TGC CCA TGG CAG GCC GTG CTG ATA AAT GAG AAG GGG GAA GAG TTT TGT GGC GGA ACT    810
241 Ile Val Gly Gly Asp Glu Cys Arg Pro Gly Glu Cys Pro Trp Gln Ala Val Leu Ile Asn Glu Lys Gly Glu Glu Phe Cys Gly Gly Thr    270

811 ATA CTG AAT GAA GAT TTC ATC CTT ACT GCT GCT CAT TGC ATA AAC CAA TCC AAA GAG ATC AAA GTT GTT GTT GGT GAA GTG GAT AGA GAA    900
271 Ile Leu Asn Glu Asp Phe Ile Leu Thr Ala Ala His Cys Ile Asn Gln Ser Lys Glu Ile Lys Val Val Val Gly Glu Val Asp Arg Glu    300

901 AAG GAA GAA CAT TCT GAA ACA ACA CAT ACT GCA GAA AAA ATA TTT GTT CAC TCT AAG TAC ATC GCC GAG ACT TAT GAT AAT GAC ATA GCC    990
301 Lys Glu Glu His Ser Glu Thr Thr His Thr Ala Glu Lys Ile Phe Val His Ser Lys Tyr Ile Ala Glu Thr Tyr Asp Asn Asp Ile Ala    330

991 CTC ATA AAG CTG AAG GAA CCC ATA CAG TTT TCG GAG TAT GTT GTC CCA GCA TGC CTC CCA CAA GCA GAC TTT GCT AAT GAA GTG CTG ATG   1080
331 Leu Ile Lys Leu Lys Glu Pro Ile Gln Phe Ser Glu Tyr Val Val Pro Ala Cys Leu Pro Gln Ala Asp Phe Ala Asn Glu Val Leu Met    360

1081 AAC CAA AAG TCT GGG ATG GTT AGT GGC TTT GGG CGT GAA TTT GAA GCT GGA CGG CTT TCC AAA AGA CTG AAA GTG CTC GAA GTC CCC TAT   1170
 361 Asn Gln Lys Ser Gly Met Val Ser Gly Phe Gly Arg Glu Phe Glu Ala Gly Arg Leu Ser Lys Arg Leu Lys Val Leu Glu Val Pro Tyr    390

1171 GTT GAT AGG AGC ACT TGC AAG CAG TCC ACT AAC TTT GCA ATA ACA GAA AAC ATG TTC TGT GCT GGT TAT GAA ACA GAG CAA AAG GAT GCT   1260
 391 Val Asp Arg Ser Thr Cys Lys Gln Ser Thr Asn Phe Ala Ile Thr Glu Asn Met Phe Cys Ala Gly Tyr Glu Thr Glu Gln Lys Asp Ala    420

1261 TGT CAA GGA GAC AGT GGA GGC CCC CAT GTA ACC AGA TAT AAG GAT ACT TAC TTT GTT ACT GGA ATT GTT AGC TGG GGA GAA GGA TGT GCA   1350
 421 Cys Gln Gly Asp Ser Gly Gly Pro His Val Thr Arg Tyr Lys Asp Thr Tyr Phe Val Thr Gly Ile Val Ser Trp Gly Glu Gly Cys Ala    450

1351 AGG AAG GGC AAA TAC GGT GTA TAT ACC AAG CTG TCC AGA TTC TTA CGT TGG GTA AGG ACA GTC ATG AGG CAA AAG TAG TAG TGG CGT GGC   1440
 451 Arg Lys Gly Lys Tyr Gly Val Tyr Thr Lys Leu Ser Arg Phe Leu Arg Trp Val Arg Thr Val Met Arg Gln Lys                        475

1441 TCT CAA ACC TCC CAT TAG GAA ACA AGG TGC AGT TTC TCT AAT GGA AAT GTT TTT TAT TTG TAT TTT TTA GCC ACA TCT GCT AAA CTT GTT   1530
1531 TTG AAA GCT GGT TTC TTG AAG TTA TGG TGC TGC TTC TGT TTT CTT TCT TTT ATT TTC TTG ACT TTA AGC ATG TAT ATG TGG TCT GCT GGT   1620
1621 GGT GTT TTG AGT TTG TAC AGT AAG GGG CTT TCC GCC AAA CAA AGT GCC TGC TCA TTG GTG TCT TCT GAT TGC TTG ACT GGT TAT TTG CAT   1710
1711 AGT GTC ATT CTG GTT CCT GTC TGT GCC CAA GTG ATT CCA CCA GAG GCT CCT GGA CAT AAC ATG ATG GAA GTG GGA AGG CCA GGA CTT CAG   1800
1801 CCA GTC CTC CTA CTG CAG CCC ACT CAC TCA GTA GGG AGT TTG TTC CCA CTG GAG CTT TTT AGC TTC TAC CTG TGG CTG TCC TTG GAA GAT   1890
1891 GAA GGG AGA AGG GAA GCT TCC TCG GCC AAA GCT GAG ACA CAA GTA AGT ATT CAG TGT GTT GCT AAA CAA CCA CGT TCC ACA AAA CTT CCC   1980
1981 AGT TAA GTC ACA GCA GCT AAA TAA AGT GTT GTG TTG CTT TAT ATG GTT GTA CTT CAC TCT AGT TTA ATT TGA ACA CTT ATT TTC TGC TCC   2070
2071 TTC AGA AAA AAA TAA CAT GTA TGT TCA CTG TTT CCT GTA TTA TAT TCT GGA TTG TGC ATG GCT GAG AAT CGG TCC TTC TGT GTG TAG TTG   2160
2161 TTC CAG GTG GTA CCG TAA GAT GCA TTA TCA GTG TA----3'                                                                   2195
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Fig. 1. Nucleotide sequence of cloned cDNA encoding the virus activating protease (VAP) precursor. The predicted amino acid sequence is shown below the nucleotide sequence. The two underlines indicate the amino acid sequences previously determined [7]. The nucleotide sequence was obtained from the clone pCHP21 because it was the longest one obtained.

translational initiation site was assigned to the methionine codon composed of nucleotide residues 1–3, because the eukaryotic translation initiation site consensus sequence, CCACCATGG [14], exists surrounding this ATG. The translation termination codon (TAG) occurs in a frame at nucleotide residues 1426–1428. Thus, VAP consists of 475 amino acids and its calculated molecular weight is 53139. The polyadenylation signal AATAAA exists at nucleotide residues 2000–2005.

3.2. *Identity of VAP with the blood clotting factor Xa*

Amino acid homology search for the VAP sequence revealed a high homology with BFX or HFX. The alignment in Fig. 2 indicates that VAP consists of the prepro leader peptide (amino acid residues 1–40), the light chain (41–185), the activation peptide (186–240) and the heavy chain (241–475). Sequence information already available for the N-termini of light and heavy chains and preservation of paired basic amino acid residues for proteolytic processing suggest strongly that VAP undergoes processing analogous to that of BFX or HFX [15]. The prepro leader peptide appears to be removed by two processing steps, one by signal peptidase cleaving after one of an upstream small residue such as Ala-20, Ser-30 and Ala-31, and the other by a second protease cleaving Arg-40 on the carboxy end of the leader peptide. The single chain precursor without prepro leader peptide is then processed into a two-chain molecule by cleavage between Arg-185 and Ser-186. This two-chain molecule is previously designated pVAP [7] and corresponds to FX. Finally the two-chain molecule is converted to the enzymatically active VAP corresponding to FXa by cleavage between Arg-240 and Ile-241 to remove the activation peptide. The calculated molecular weight of pVAP, VAP and activation peptide are approximately 49000, 43000 and 6000, respectively. These values are in good agreement with

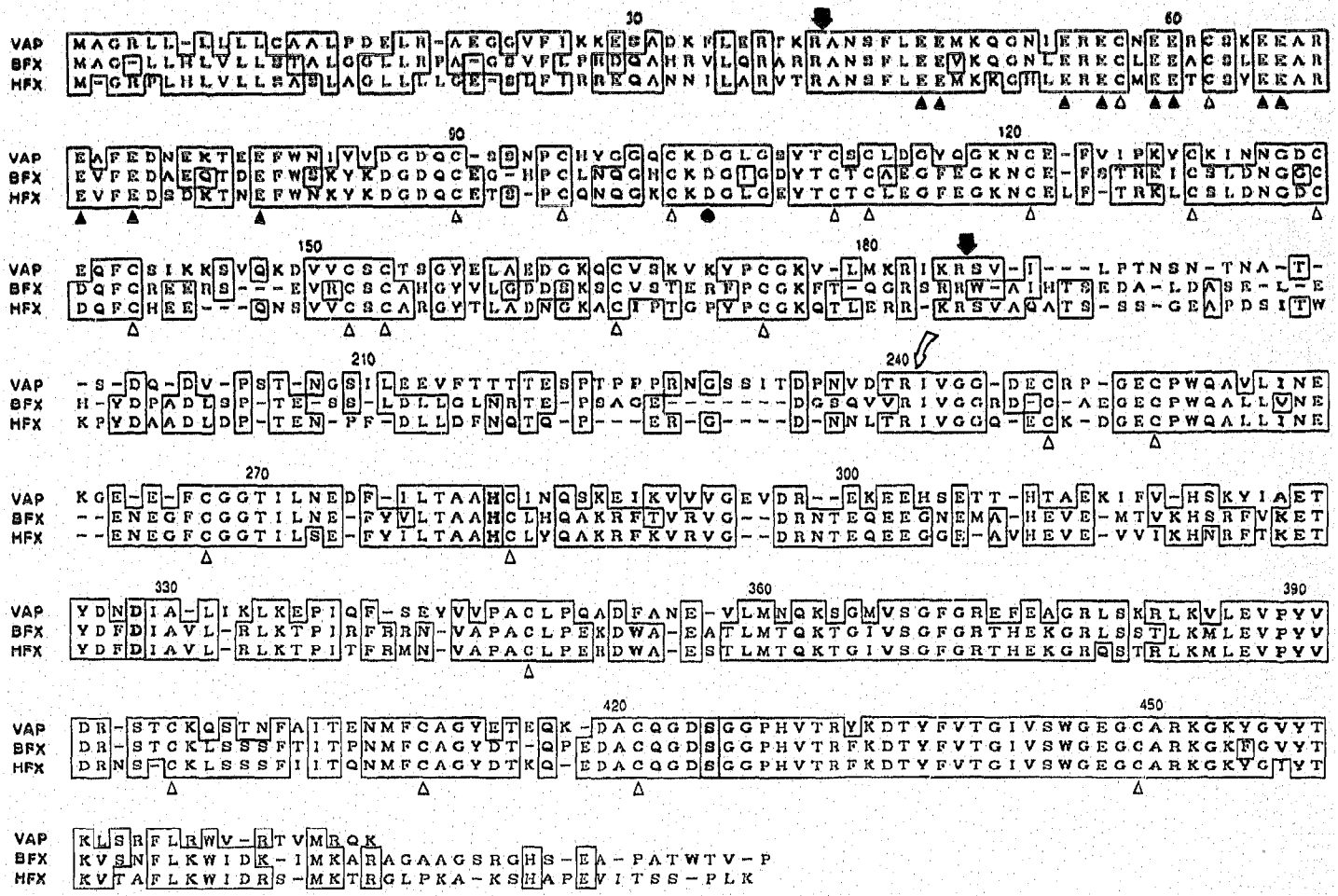

Fig. 2. Alignment of the amino acid sequence of the prepro form of VAP (top), BFX (middle) and HFX (bottom). Identical amino acids at the same position are boxed. Gaps have been introduced to maximize the homology. The two solid arrows are possible processing sites and the open arrow is the cleavage site for activation. The solid arrowheads and solid circle indicate presumable γ-carboxylated residues and a β-hydroxylated residue, respectively. All cysteine residues conserved in the three sequences are indicated by open arrowheads. The catalytic triads are in dotted boxes. The sequence data for BFX and HFX are from [21–24].

the molecular mass estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (70 kDa, 55 kDa and 18 kDa, respectively) [7], assuming that the potential N-glycosylation sites are actually utilized at residues 196, 207, 228 and 285.

Fig. 2 further shows that essentially all functional and structural domains and residues characteristic of BFX or HFX are conserved in VAP. In the light chain of BFX and HFX, the eleven glutamic acid residues in the N-terminal region are known to be posttranslationally converted to γ-carboxyglutamic acids (Gla) in a vitamin K-dependent process, and serve as Ca^{2+} binding sites [16]. VAP also requires Ca^{2+} for virus activation and its N-terminal three glutamic acid residues were already suggested to be γ-carboxylated [7]. The strict conservation of those three and the additional eight glutamic acid residues suggests that they are indeed γ-carboxylated and play a crucial role in Ca^{2+} binding. After this Gla domain, two potential growth factor domains exist. The cysteine residues important for this domain formation are well conserved at positions 90, 95, 101, 110, 112, 121, 129, 136, 140, 152, 154, 167, and cysteine-175 is likely involved in the interchain bond together with cysteine-348 in the heavy chain. The aspartic amino acid conserved at position 103 could be β-hydroxylated [15]. In the heavy chain, the three principal amino acids (His-282, Asp-328 and Ser-425) participating in catalysis (the catalytic triad) are completely preserved. In addition, all predicted amino acid residues which effect substrate binding of FXa [17], residues 266, 325, 372, 420, 422, 447, 448 and 450 are strictly conserved in the three sequences. Furthermore, the cysteine residues crucial for domain formation of the catalytic heavy chain are also completely conserved at positions 247, 252, 267, 283, 348, 396, 410, 421 and 449.

These results strongly suggest that VAP is identical with CFXa and pVAP corresponding to CFX. Further substantiating this, a cDNA clone with the identical deduced amino acid sequence was obtained by using the same oligonucleotide probes from adult fowl liver where FX is known to be constitutively synthesized (data not shown).

3.3. *Expression of VAP mRNA in both the CAM and the liver of chick embryo*

In higher organisms, FX is synthesized in the liver as one of the zymogens in the blood clotting cascade. To further confirm the identity between VAP and CFXa, expression of VAP-specific mRNA in the liver was investigated by blot hybridization analysis of poly(A)$^+$ RNA. A 3′-non-coding region of VAP cDNA was used as a specific probe, and hybridization was performed under the strict condition as described in section 2. A discrete signal was observed at exactly the same position in both the embryonic and the adult liver as well as in CAM (Fig. 3). The estimated size of the RNAs

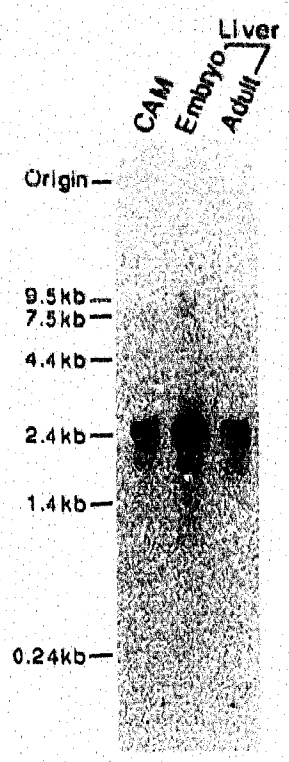

Fig. 3. Blot hybridization analysis of chick poly(A)$^+$ RNA from CAM, embryonic liver and adult liver. The amount of each poly(A)$^+$ RNA used was 5 μg. Autoradiography was performed at −70°C for 48 h with an intensifying screen.

was approximately 2.5 kb and therefore well-matched to the cDNA size.

4. DISCUSSION

In this paper, we cloned and sequenced a cDNA specific for the mRNA encoding VAP of chick embryo, and demonstrated that VAP is, by all molecular biological criteria employed, identical with FXa. Both the successful isolation of cDNA from the CAM and the blot hybridization analysis of poly(A)$^+$ RNA giving a clear, specific signal for the CAM indicate an ectopic expression of FX in this organ. In addition, by specific immunofluorescent staining, we detected the FX molecule in the endodermal cell layer of the CAM, which is in direct contact with the allantoic fluid (manuscript in preparation). Therefore, it is a reasonable assumption that FX is synthesized in the endoderm of the CAM, secreted into the allantoic fluid and converted to the active FXa. In mammals, the processing of FX to FXa is done by factor IXa or factor VIIa at a middle phase of the blood clotting cascade [16]. The enzymes responsible for this conversion in the allantoic cavity are not known. Also to be elucidated are the physiological roles beside clotting, which the FX/FXa may play in chick embryogenesis. In this

Table I

Amino acid sequence homology in each structural domain of FX among three species

	Chick vs. bovine (%)	Chick vs. human (%)	Bovine vs. human (%)
Prepro leader peptide	48	44	51
Light chain	58	62	69
Activation peptide	29	33	41
Heavy chain	65	65	79
Whole sequence	58	59	69

respect, it is worthy to note that prothrombin or prothrombin-like proteases are possibly involved in the morphological differentiation of human neuroepithelial cells [18] and in the dorsal-ventral pattern formation of *Drosophila* embryos [19,20].

Table I compares the amino acid homology of FX among three species. The heavy chain is the most homologous, suggesting a strong functional and structural constraint exerted on this domain in the evolutionary process. This high degree of conservation in the entire catalytic chain would be the basis conferring a highly specific substrate recognition on FXa, because the entire molecular surface surrounding the catalytic pocket is highly characteristic of FXa but is different from those of other related serine proteases, while the catalytic pocket is widely preserved among these different serine proteases [17]. It has to be noted that the viral fusion glycoprotein to be cleaved by the FXa shares a consensus tripeptide sequence of the cleavage site with prothrombin [7]. The light chain appears to be less homologous than the heavy chain, but all the functionally and structurally important residues such as those for the Gla domain and the growth factor domain are strictly preserved. On the other hand, the lowest degree of conservation for the activation peptide, previously noticed between HFX and BFX [23], was much more drastically shown in the present comparison, indicating a high evolutionary rate of this region because of its low constraint. The prepro leader peptide appears to maintain a considerable homology, apparently higher than that generally found among signal peptides for membrane insertion. A significant number of conserved residues were further identified in this region of the other vitamin K-dependent blood clotting factors [16]. Thus, the homologous prepro leader peptides play some role in γ-carboxylation.

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