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N-terminal domains of putative helicases of flavi- and pestiviruses may be serine proteases

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ABSTRACT

Recently we tentatively identified, by sequence comparison, central domains of the NS3 proteins of flaviviruses and the respective portion of the pestivirus polyprotein as RNA helicases (A.E.G. et al., submitted). Alignment of the N-proximal domains of the same proteins revealed conservation of short sequence stretches resembling those around the catalytic Ser, His and Asp residues of chymotrypsin-like proteases. A statistically significant similarity has been detected between the sequences of these domains and those of the C-terminal serine protease domains of alphavirus capsid proteins. It is suggested that flavivirus NS3 and the respective pestivirus protein contain at least two functional domains, the N-proximal protease and the C-proximal helicase one. The protease domain is probably involved in the processing of viral non-structural proteins.

INTRODUCTION

Most eukaryotic positive strand RNA viruses have two or three non-structural proteins whose sequences, and presumably structures, are highly conserved. These include RNA-dependent RNA polymerases (1,2), NTP-binding motif-containing proteins predicted to be RNA helicases (3-5), and proteases (6,7). Recently we tentatively identified, by sequence comparison, helicase-like domains in the C-terminal one-halves of flavivirus NS3 proteins and in the similar portion of the pestivirus polyprotein (A.E.G. et al., submitted). Genome expression of both groups of viruses occurs by polyprotein processing (7-9). Specifically it has been hypothesized that flavivirus non-structural proteins NS3, NS5 and NS2B are released from the polyprotein by a virus-encoded protease cleaving after basic residues, mainly R(K)R dipeptides (8,10-12). However, no data pertaining to identification of this protease with one or another of viral proteins is available. As no similarity between any flavivirus protein sequence and serine proteases was detected, it was suggested that the protease involved might be of the thiol type (8). Here, we show that the N-terminal domains of flavivirus NS3 proteins and the respective portion of the pestivirus polyprotein do contain conserved segments similar to those around the catalytic residues of serine chymotrypsin-like proteases and display a statistically significant sequence similarity to the protease domains

of alphavirus capsid proteins. We suggest that NS3 proteins and the homologous pestivirus protein contain at least two functional domains, the N-proximal protease and the C-proximal helicase one.

METHODS

Amino acid sequences

Amino acid sequences compared were those of NS3 proteins of flaviviruses: yellow fever (YFV), West Nile (WNV), Dengue types 2 (DEN2) and 4 (DEN4), Japanese encephalitis (JEV), Murray Valley encephalitis (MVE) and Kunjin (KUN); polyprotein of bovine viral diarrhea virus (BVDV, a pestivirus); capsid proteins (CP) of alphaviruses: Sindbis (SNBV), Semliki forest (SFV), Ross River (RRV), Eastern equine encephalitis (EEEV), Venezuelan equine encephalitis (VEEV) and Western equine encephalitis (WEEV). Sequences were from current literature (see Fig.1A).

Sequence comparisons

Amino acid sequences were compared by programs DIAGON (13) and OPTAL (3,14), using the amino acid residue comparison matrix MDM78 (15). Program OPTAL, based on the original algorithm of Sankoff (16), performs stepwise optimal alignment of multiple amino acid sequences and its statistical assessment by a Monte Carlo procedure. Adjusted alignment score (AS) is calculated in standard deviation (SD) units:

$AS = S^o - S^r/\sigma$

where S^o is the score observed upon comparison of real sequences, S^r is the mean score obtained upon intercomparison of 25 randomly jumbled sequences (or sets of aligned sequences) identical to the real ones in amino acid composition, and σ is the standard deviation. Program DIAGON was written in the C programming language and run on a WICAT S150 computer. Program OPTAL was written in FORTRAN 77 and run on an IBM PC AT.

RESULTS AND DISCUSSION

Identification of putative flavivirus and pestivirus proteases and of their functional sites

Comparison of the amino acid sequences of flavivirus and pestivirus polyproteins revealed considerable similarity which was most prominent in NS5 and central parts of NS3 proteins of flaviviruses (17), i.e. putative RNA polymerases and putative helicases, respectively (A.E.G. et al., submitted). Comparison by the DIAGON program (not shown) revealed that the latter similarity extended into the N-terminal region of NS3. Alignment of the respective flavivirus and pestivirus sequences by program OPTAL yielded AS values of about 3 SD which is considered only conditionally significant (15,18). Unexpectedly, however, inspection of the derived pattern of conserved residues (Fig.1A) revealed the sequence GxSGxP (x, any residue) strikingly resembling the conserved motif around the catalytic S residue of serine chymotrypsin-like proteases (19,20). This prompted a more detailed detailed comparison of the sequences of these domains to those of serine proteases. Upon such a comparison, a statistically significant similarity has been detected to the C-terminal protease domains of alphavirus capsid proteins, with AS of about 4.5 SD

(A)

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No      Ref       1          11         21         31         41         51
                                                                            *
 1 VEEV (29): 102 GnkkktNkkp GKrqRmVMKL ESdkTFpIML e---GKiN-G YAcVVGGkLf rpmHV-eGKI
 2 SNBV (30):  94 -pA---kpkp GKrqRmALKL EadrLFdVKN ed--Gdvi-G hALAMEGkVm kplHV-KGtI
 3 SFV  (31):  95 kqAdkkkkkp GKreRmCMKI ENdcIFeVKh e---GKvT-G YAcLVGDkVm kpaHV-KGvI
 4 RRV  (32): 101 -qA--kkkkp GRreRmCMKI ENdcIFeVKL d---GKvT-G YAcLVGDkVm kpaHV-KGtI
 5 EEEV (33):  91 r-----kpkp GKrqRmCMKL ESdkTFpIML n---GqvN-G YAcVVGGrVf kplHV-eGRI
 6 WEEV (34):  89 r-----kpkp GKrqRmCMKL ESdkTFpIML n---GqvN-G YAcVVGGrLm kplHV-eGKI

 cons1                    kp GKrqRm MKL E d  F I       G v  G  A  V  kV  kp+HV  G I
                              R e   L I        V         i        M  rL  r
                             ..  .  ... .    . :       |    |  :  ..  .     |.  | .
 cons2                    P                    I       G    G  G   EG    T+ H T G
                                               L               A   Q     S

 7 BVDV: (35)  ?  ltAfFgiMPr GttpRapVRf p---TslLKV Rr--GleT-G WAytHQGgIs SVdHVTaGKd

 8 YFV : (10)  0  sgdvlwDIPt pKiieeCehL EDg-IYgIfQ stflGasQrG vGVaqGGvfh TMwHVTRGaf
 9 WNV : (36)  0  Gg-vlwDtPs pKeyKkg-dt tTg-VYrIMt RgllGsyQaG aGVMVEGvfh TLwHtTKGaa
10 KUN : (11)  0  Gg-vlwDtPs pKeyKrg-dt tTg-VYrIMt RgllGsyQaG aGVMVEGvfh TLwHtTKGaa
11 JEV : (37)  0  Gg-vFwDtPs pKpcskg-dt tTg-VYrIMa RgilGtyQaG vGVMyENvfh TLwHtTRGaa
12 DEN4: (38)  0  sg-alwDVPs paatKkA-aL sEg-VYrIMQ RglfGKtQvG vGIhMEGvfh TMwHVTRGsV
13 DEN2: (39)  0  Ag-vlwDVPs pppvgkA-eL EDg-aYrIKQ KgilGysQiG aGVykEGtfh TMwHVTRGaV
14 MVE : (40)  0  Gg-vFwDtPs pKvypkg-dt tpg-VYrIMa RgilGRyQaG vGVMHEGvfh TLwHtTRGaa


     61         71         81         91         101        111        121        131
                            *
 1 : dnd----vla aL-kTkkasK yD-LEYAdvp QnmRaD-tfk YthEkpqGyy ---swhhgAV QyenGrFTVP kG
 2 : dhp----vlS KL-kFTkSsa yD-MEFAqlp vnmRsE-AfT YtsEhpeGfy ---nwhhgAV QySGGrFTIP rG
 3 : dna----Dla KL-aFkkSsK yD-LEcAqip vhmRsD-Ask YthEkpeGhy ---nwhhgAV QySGGrFTIP TG
 4 : dnp----Dla KL-tYkkSsK yD-LEcAqip vhmKsD-Ask YthEkpeGhy ---nwhhgAV QySGGrFTIP TG
 5 : dne----Qla aI-klkkasI yD-LEYGdvp QcmKsD-tlQ YtsDkppGfy ---nwhhgAV QyennrFTVP rG
 6 : dne----Qla aV-klkkasM yD-LEYGdvp QnmKsD-tlQ YtsDkppGfy ---nwhhgAV QyenGrFTVP rG

     d       l   +    k s  yD LE Gd+p   mR D     Yt E p G y     whhgAV Qy   rFTIP  G
                              M  Aq      K E        D                          V
                ..          | ...:       . .     .  :   .           .: |     |...  :
     L+         R+          D   YG                 VQ   +E           + Q     F     G
     I          K                                  LN    N                         A

 7 : LLvcdsmGrT RVVcqSnNrL tDetEYGvkt DsGcpDgARc YvLNpeAVNi sgsKgAVVHL QkTGGeFTcV TA

 8 : LVr----Ngk KLIpSwaSvK eDlVaYGgsw klegrw-dgE eeVQliAAvp ---gknVVnV QtkpslFkVr nG
 9 : LMs----Geg RLdpYwgSvK eDrLcYGgpw klqhkw-ngh deVQmivVEp ---gknVknV QtkpGvFktP eG
10 : LMs----Geg RLdpYwgSvK eDrLcYGgpw klqhkw-ngQ deVQmivVEp ---gknVknV QtkpGvFktP eG
11 : IMs----Geg KLtpYwgSvK eDrIaYGgpw rfdRkw-ngT ddVQvivVEp ---gkAaVnI QtkpGvFrtP fG
12 : Ich----Etg RLepSwaDvR nDmIsYGggw rlGlkw-dKE edVQvlAIEp ---RknpkHV QtkpslFktL TG
13 : LMh----kgk RIepSwaDvK kDlIsYGggw klegew-keg eeVQvlALEp ---gknprAV QtkpGlFrtn TG
14 : IMs----Geg RLtpYwgNvK eDrVtYGgpw kldqkw-ngv ddVQmivVEp ---gkpaInV QtkpGiFkta hG


              141        151        161        171        181        191        201
                     *
 1 : --VGA--- ----KGdSGr PILD-nQGRV VAIVlgGVNE GSR--TaLSv VMwnEKgVtv KYtp--ENce qw-  0
 2 : --VGG--- ----RGdSGr PIMD-nSGRV VAIVlgGaDE GTR--TaLSv VTwnSKgkti KTtp--EgTe ew-  0
 3 : --aGk--- ----PGdSGr PIFD-nKGRV VAIVlgGaNE GSR--TaLSv VTwn-KDMvt Rvtp--EgSe ew-  0
 4 : --aGk--- ----PGdSGr PIFD-nKGRV VAIVlgGaNE GaR--TaLSv VTwT-KDMvt Rvtp--EgTe ew-  0
 5 : --VGG--- ----KGdSGr PILD-nKGRV VAIVqgGVNE GSR--TaLSv VTwnQKgVtv KDtp--EgSe pw-  0
 6 : --VGG--- ----KGdSGr PILD-nRGRV VAIVlgGVNE GTR--TaLSv VTwnQKgVti KDtp--EgSe pw-  0
```

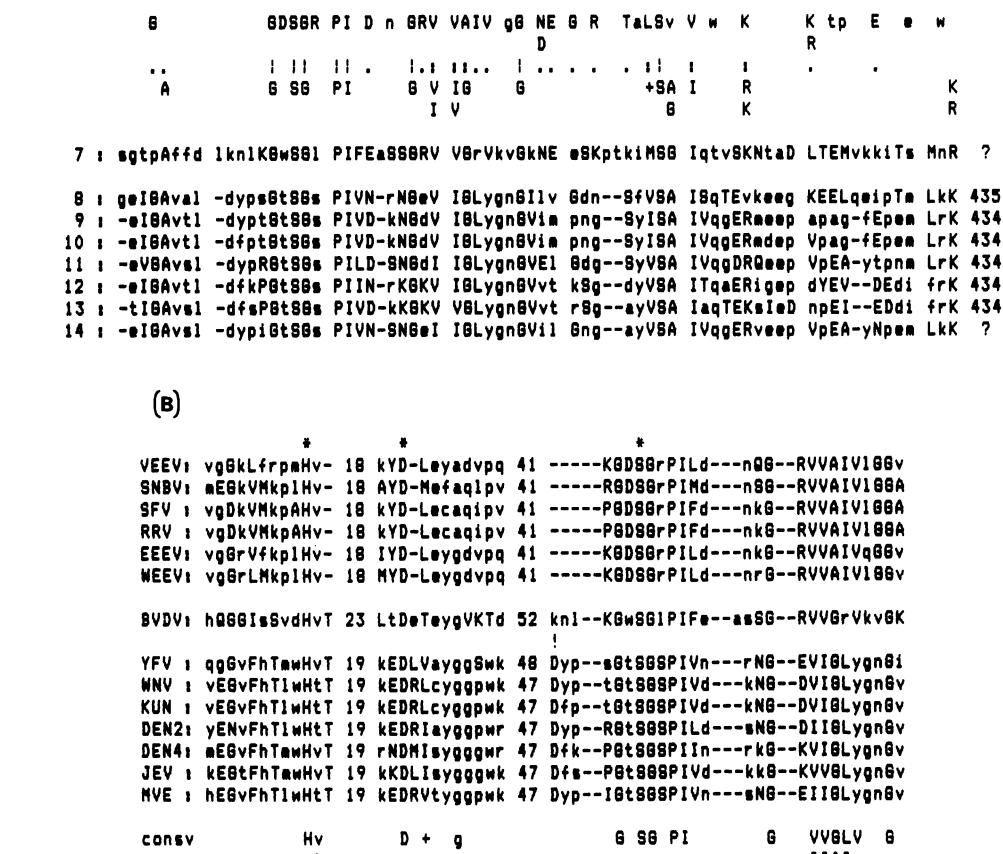

Fig.1. Putative proteases of flavi- and pestiviruses.
(A) Alignment of the amino acid sequences of the N-terminal domains of flavivirus NS3 proteins, the similar portion of the p125 protein of BVDV and the C-terminal domains of alphavirus CP. The abbreviations of viruses stand for respective proteins. The distances to the protein termini are indicated by numbers. The aligned sequences of NS3 begin from the protein N-termini, and those of CP extend to the C-termini. For BVDV, protein bounderies are unknown. The segment shown is from residue 1701 to 1897 of the polyprotein. For MVE, only a partial sequence

for the alignment of the BVDV sequence with CP, and about 5 SD for NS3 vs. CP. Perhaps more importantly, for most of the residues conserved between the proteins of flavi- and pestiviruses, counterparts were observed in the consensus pattern for the alphavirus CP, or, at least, respective residues were observed in some of the CP sequences (Fig.1A). Most prominent was the similarity between the two alignments around the putative catalytic S residues, and invariant H and D residues also could be detected (Fig.1A). A number of similarities was observed when the sequence stretches of viral proteins around the putative catalytic residues were aligned with the respective sequences of chymotrypsin-like proteases; also, the spacing of these residues was generally compatible in both groups of proteins (Fig.1B). Thus, it is likely that the relationship between the central parts of NS3 and the respective pestivirus domain, and the protease domains of alphavirus CP is authentic and that the former also possess protease activity.

An important corollary of these observations is the tentative re-identification of the catalytic D residue of alphavirus

Fig. 1 legend continued ....

has been reported. The numbering above the alignment is arbitrary, beginning from the first aligned residue. Capitals highlight identical and similar amino acid residues in proteins of of different groups of viruses. Residues within the following groups were considered similar: L,I,V,M; S,T; G,A; R,K; D,E,N, Q; F,Y,W. Cons1, consensus pattern of residues conserved in alphavirus CP (correspondence in all included sequences was required); cons2, consensus pattern for flavi- and pestivirus proteins (an exception in one of the flavivirus sequences was allowed). +, hydrophobic residues (L,V,I,M); Vertical streaks designate complete correspondence between cons1 and cons2, and colons partial correspondence; dots highlight positions where no consensus could be derived for one of the protein sets but the consensus for the other was met in at least one of the sequences. Asterisks denote putative catalytic residues. Source references are indicated in parentheses before each of the aligned sequences.
(B) Alignment of sequence stretches around the (putative) catalytic residues of selected chymotrypsin-like proteases, alphavirus CP and similar proteins of flavi- and pestiviruses. Additional abbreviations: CHT, chymotrypsin; TRP, trypsin; RPE, rat pancreatic elastase; HNE, human neutrophil elastase; THR, thrombin; SGPA, Streptomyces griseus protease A; ALP, bacterial α-lytic protease. Chymotrypsin-like protease sequences were from (41), except HNE which was from (42). Residues identical or similar in viral and cellular proteins are highlighted by capitals. Consv, consensus pattern of residues conserved in (putative) viral proteases; consc, consensus pattern for cellular proteases. Both patterns were derived allowing one exception. Numbers stand for the distances between the conserved segments. '!' denotes the residue responsible for primary cleavage specificity in chymotrypsin-like proteases, and possibly in flavivirus proteases (see text). Other designations are as in (A).

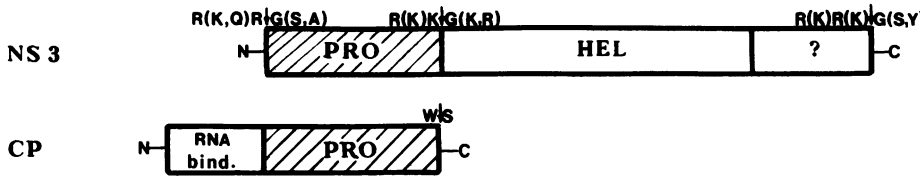

Fig.2. Domain organization of alphavirus CP and flavivirus NS3 proteins.
Proteins are designated by rectangles drawn to scale. Regions displaying sequence similarity (hatched; cf. Fig.1A) are aligned. PRO, (putative) protease domain; HEL, putative helicase domain; ?, domain of unknown function. The boundaries of the helicase domain could be derived from sequence comparison of putative viral helicases (A.E.G. et al., submitted) N- and C-termini of the proteins and residues flanking the cleavage sites are indicated; also shown is the putative internal cleavage site in NS3.

CP. Previously, another D residue conserved in these proteins [D^{61} in Fig.1A, or D^{147} in the original alignment (8)] was implicated in catalysis based on its conservation and on interpretation of mutagenesis data (8, 21). D^{82} (D^{163} in the original alignment) which is conserved in the alignment with the flavivirus and pestivirus proteins (Fig.1A) is, however, a more plausible candidate for the catalytic residue as its location relative to the conserved H residue corresponds much better to that in chymotrypsin-like proteases (Fig.1B). Also, implication of D^{82} in catalysis better explains the location of the mutation ts128 which results in substitution of S for P^{90} [P^{170} in the original alignment (8)]. These observations may eventually lead to re-evaluation of the structural model suggested for the alphavirus CP by Fuller and Argos (22).

Inspection of the alignment of (putative) proteases of alpha-, flavi- and pestiviruses allows tentative prediction not only of the catalytic residues but also of the substrate-binding pocket. Based on the analogy with chymotrypsin-like proteases (23,24), we suggest that the walls of this cavity may be partially constituted by the highly conserved segments C-terminal of the proposed catalytic S residues; specifically, the invariant residues G^{167} and S^{179} (Fig.1A) might be involved.

The cleavage specificity of chymotrypsin-like proteases appears to be determined primarily by a region preceding, in the polypeptide chain, the catalytic S residue and constituting the bottom of the substrate-binding pocket (23,25). The specificities of the alphavirus and of the putative flavivirus proteases are apparently different. Whereas the capsid proteases cleave after a W residue, similarly to chymotrypsin (8), cleavage of flavivirus non-structural proteins probably affected by the NS3 protease occurs after basic residues, resembling the specificity of trypsin (see Introduction). The specificity of the putative protease of BVDV is totally obscure, and no sequences similar to cleavage sites of any known protease could be found near the proposed protein boundaries (9,17). In accord with the observed specificities, D^{142} conserved in flavivirus NS3 may confer the

affinity towards basic residues (see also Fig.1B), whereas the conserved G^{136} of the alphavirus CP, as a small residue might be responsible for the observed specificity to bulky W residues.

Functional and evolutionary implications

The presented data indicate that flavivirus NS3 proteins and the homologous protein (p125) of BVDV may contain two domains, the N-proximal protease and the C-proximal RNA helicase one. It is interesting to compare the domain organization of flavivirus NS3 to that of the alphavirus CP (Fig.2). Proteins of both groups appear to have a protease and RNA-binding domains [in CP, the latter is involved in the RNA-protein interaction within the nucleocapsid (22)] but their orientation is opposite in each case Significantly, the similarity between CP and NS3 extended exactly to the interdomain boundaries (Fig.2) which could be determined from the respective alignments [(21) and A.E.G. et al., submitted]. In NS3, a fully conserved dipeptide R(K)K is found at this boundary (Fig.2), raising the interesting possibility of an alternative cleavage pathway, with NS3 autolytically cleaved into two separate smaller proteins with the protease and the helicase activities. A directed search for such proteins in flavivirus-infected cells seems worthwhile. The possibility of the protease and the helicase domains residing in some cases in multidomain, and in others in separate proteins, seems even more likely for BVDV. The reported cleavage of p125 into p54 and p80 probably occurs between these two domains (cf.9,17); apparently, the latter two proteins have, respectively, N- and C-terminal domains, in addition to the putative protease and helicase ones. On the other hand, the cleavage of p125 seems not to take place in cells infected with non-cytopathic strains of BVDV (9).

Besides flavi- and pestiviruses, three groups of positive strand RNA viruses appear to possess both a helicase and a protease(s), namely alpha-, poty- and coronaviruses (3,43). In poty-, and probably in coronaviruses, the localization of the respective domains of the polyproteins relative to each other is quite different from that described here for flavi- and pestiviruses, and they reside in different mature proteins. In alphaviruses, the processing of the non-structural proteins is mediated by a virus-encoded protease other than CP (7,8) and, according to recently reported preliminary data (cf.7), this activity has been identified with nsP2 protein encompassing also the putative helicase domain (3-5). NsP2 has, in addition to the helicase domain, N- and C-terminal domains of which each could, in principle, comprise the protease activity. Thus, this alphavirus protein may be analogous to flavivirus NS3 in having a helicase and a protease domains. Careful inspection of the sequences of the N-terminal regions of nsP2 revealed some stretches of marginal similarity to the segments of chymotrypsin-like proteases around the catalytic residues (not shown) but identification of this alphavirus protease requires further detailed analysis.

Finally, it is interesting to note that the presence of a protease and an NTPase (e.g. helicase) domains within one protein, as described here, is not without precedent outside the viral world. Specifically, this is probably the case for the La protease of E.coli mediating ATP-dependent degradation of abnormal polypeptides (26,27). Interestingly, both the ATPase and the protease activities of this protein are stimulated by DNA (28), enhancing the case for a functional similarity to helicases. The

ATPase domain of La encompasses the NTP-binding structural motif (27) conserved, among other NTP-utilizing enzymes, in the putative helicases of positive strand RNA viruses [(3-5) and A.E.G. et al., submitted]. Moreover, some additional local sequence similarity, beyond the motif formula, is observed between La and the latter proteins (unpublished data). In this connection, an intriguing speculaton amenable to experimental test is that processing of flavi- and pestivirus polyproteins might be an ATP-dependent process.

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