Recombinant baculovirus-expressed NS3 proteinase of hepatitis C virus shows activity in cell-based and in vitro assays

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Recombinant baculoviruses have been constructed which express the hepatitis C virus (HCV) NS3 proteinase and its substrates in insect cells. The expressed proteinase has been shown to carry out trans-cleavage at the NS3/4A, NS4A/4B, NS4B/5A and NS5A/5B junctions in a cell-based assay. When assayed in a cell-free system using in vitro translated substrates, the proteinase could perform trans-processing of the NS4A/4B and NS5A/5B junctions, but only when co-expressed with NS4A, either as an NS3–4A precursor or by co-infection of cells with NS3- and NS4A-expressing recombinant baculoviruses. Possible reasons for the absolute requirement of the NS3 proteinase for NS4A in vitro are discussed.

Introduction

The genome of the hepatitis C virus (HCV) is a single-stranded RNA molecule of positive sense, which encodes a single open reading frame capable of expressing a polyprotein of approximately 3000 amino acids (Kato et al., 1990; Choo et al., 1991). In the absence of a satisfactory cell culture system for propagating the virus, studies of its mode of gene expression have depended upon the use of heterologous systems for protein expression, including in vitro translation, transient expression in mammalian cells and production of recombinant vaccinia viruses. By these methods, it has been established that the NS3 region of the polyprotein contains a serine proteinase domain which is responsible for cleavage of the downstream polyprotein in at least four places (Bartenschlager et al., 1993, 1994; Eckart et al., 1993; Grakoui et al., 1993; Hijikata et al., 1993; Tomei et al., 1993; Manabe et al., 1994). By analogy with the yellow fever virus (Chambers et al., 1990) this proteinase is probably essential for the production of infectious virus, and hence represents a possible target for chemotherapeutic intervention.

The heterologous expression systems mentioned above have the disadvantage that protein expression levels are low, making the extraction and purification of proteinase for biochemical and structural studies impracticable. The insect cell/recombinant baculovirus system, however, combines the advantages of a eukaryotic expression system with a potentially high yield of protein. This system was used by Hirowatari et al. (1993) to express a portion of the HCV non-structural region including NS3, which underwent self-cleavage at the NS3/4A junction. We describe here the construction of a range of recombinant baculoviruses, designed to express the NS3 proteinase and its substrates in insect cells. The activity of the expressed enzyme has been demonstrated in both cell-based and in vitro assays.

Methods

Cells and viruses. Spodoptera frugiperda Sf9 cells were obtained from the American Type Culture Collection. Autographa californica nuclear polyhedrosis virus (AcNPV) was provided by Dr R. Possee (Institute of Virology and Environmental Microbiology, Oxford, UK). Purified AcNPV genomic DNA was obtained from Invitrogen. Procedures for the culture of Sf9 cells and the growth and plaque assay of AcNPV have been described previously (Knudson & Tinsley, 1974; Brown & Faulkner, 1977).

Antisera. Dr R. Bartenschlager and colleagues (Hoffman-La Roche, Basle, Switzerland) kindly provided antisera against the following portions of the HCV genome expressed in Escherichia coli: amino acids 1007-1246 (anti-NS3); amino acids 1616-1738 (anti-NS3/4); amino acids 2101-2231 (anti-NS5A) and amino acids 2419-2622 (anti-NS5B) (Bartenschlager et al., 1993, 1994). A further antiserum was raised against a recombinant protein consisting of HCV amino acids 1658-1711 (NS4A) coupled to maltose binding protein and expressed in E. coli. All antisera were raised in rabbits.

Plasmid constructions. The basic plasmids pATA 1007–2234, pATA 1007–2234 S → A and pATA 1007–3011 have been previously described (Bartenschlager et al., 1994) and were kindly supplied by the authors. The plasmids consist of the modified vaccinia virus vector pATA-18, into which have been inserted parts of the HCV genome. The numbers specified refer to the first and last amino acids of the expressed HCV polyprotein fragment; in the plasmid designated S → A, the putative active site serine codon was replaced by an alanine codon.

Portions of the plasmids were subcloned to generate transfer vectors for the production of recombinant baculoviruses. The fragments of the

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Two further transfer vectors were constructed to express potential substrates for the NS3 proteinase: in order to provide the necessary initiation codons, the DNA fragments were first subcloned into the vector pRSET B (Invitrogen). pRSET 1463–2235 was constructed by digesting pATA 1007–2234 with SalI at position 4714 of the HCV sequence and XbaI in the downstream MCS. pRSET 2012–2613 was constructed by digesting pATA 1007–3011 with HindIII (position 6363) and SacI (position 8169). The two fragments were made blunt-ended and cloned into BamHI-cut, blunt-ended pRSET B, thus placing the HCV sequences in frame with an initiating ATG, followed by a region encoding a hexahistidine purification tag, and a linker region. The two pRSET plasmids were digested with XbaI and HindIII to release the HCV sequences along with the associated upstream coding sequence. The fragments were made blunt-ended and ligated into BamHI-cut, blunt-ended pAcYM1, to generate plasmids pAc.NS3'/4'/5' and pAc.NS5A'/B' respectively.

Production and analysis of recombinant baculoviruses. Recombinant baculoviruses were produced by co-transfecting the transfer plasmids and AcNPV genomic DNA into Sf9 cells using Lipofectin reagent (Gibco BRL). Recombinant virus clones were identified by dot-blot hybridization with an appropriate restriction-fragment probe (Fung et al., 1988) or, in the case of Ac.NS4A, by plaque assay in Sf9 cells and staining with X-Gal (see above). Potential recombinants were subjected to several rounds of plaque purification before analysis of their expression products as follows: monolayers of Sf9 cells were infected with viruses at a multiplicity of 5–10 p.f.u./cell and incubated at 28 °C for 24 h (previous experience has shown that expression from the polyhedrin promoter is maximal at 36–48 h post-infection, but several of our HCV recombinant viruses showed enhanced cytoxicity, leading to cell death and degradation of the expressed protein after approximately 24 h). Cells were harvested, washed with PBS and heated in protein sample buffer at 100 °C for 10 min (protein sample buffer contained 0.05 M-Tris-HCl pH 7.0, 2% SDS, 6% glycerol and 5% 2-mercaptoethanol). Samples were run on pre-cast SDS-polyacrylamide gels (Novex), blotted onto nitrocellulose and probed for immunoreactivity using the antiserum described above. Development of blots was completed using alkaline phosphate-conjugated anti-rabbit IgG (Sigma) and BCIP/NBT chromogenic substrate (Novabiochem). For blots requiring extra sensitivity, the second antibody used was biotinylated anti-rabbit immunoglobulins, followed by alkaline phosphatase-conjugated streptavidin (Dako) and BCIP/NBT substrate.

Metabolic labelling of recombinant baculovirus-infected cells. Monolayers of Sf9 cells in 35 mm dishes (1.5 × 10^6–2 × 10^6 cells per dish) were infected with recombinant viruses at a multiplicity of 5–10 p.f.u./cell and incubated at 28 °C for 18–24 h. The supernatant medium was replaced with methionine-free medium, the cells were ‘starved’ for 2 h at 28 °C and were then labelled for 1 h in methionine-free medium containing 10–15 μCi of [35S]methionine per dish. Cells were harvested, washed in PBS and lysed in 100–200 μl of RIPA buffer (0.01 M-Tris–HCl pH 8.0, 0.1% SDS, 0.15 M-NaCl, 2 mM-EDTA, 1% Triton X-100, 1% sodium deoxycholate).

In pulse-chase experiments the labelling period was reduced to 30 min, after which the supernatant medium was replaced with complete medium containing 20 μg/ml of cycloheximide. Incubation was continued at 28 °C, after which the cells were harvested in RIPA buffer as above. Cell lysates were immunoprecipitated as previously described (Matsuura et al., 1987), except that cell lysate/antiserum mixtures were held on ice. Samples were analysed by SDS-PAGE and fluorography.

In vitro assay of NS3 proteinase activity. Radiolabelled substrates were produced by using the plasmids pRSET 1463–2234 and pRSET 2012–2613 to programme a coupled transcription/translation reaction (TNT reticulocyte lysate system; Promega) according to the manu-
facturer’s protocol, in the presence of T7 polymerase and [35S]methionine. After incubation at 28 °C for 1–2 h, the translation reaction was stopped by the addition of 20 μg/ml cycloheximide. Protease was produced by infecting Sf9 cells with recombinant baculoviruses at a multiplicity of 5–10 p.f.u./cell. After incubation at 28 °C for 24 h, the cells were harvested, washed with PBS and disrupted in ice-cold assay buffer (10 mM-Tris-HCl pH 7.3, 10 mM-NaCl, 1 mM-EDTA, 5 mM-dithiothreitol, 1% Triton X-100) using 100 μl of buffer per 10^6 infected cells. The protease and substrate-containing lysates were mixed in a 5:1 ratio and held on ice for up to 5 h. Samples were analysed by SDS-PAGE and fluorography.

Results and Discussion

Expression of the HCV NS3 protease using recombinant baculoviruses

A number of recombinant baculoviruses designed to express parts of the HCV non-structural region have been produced, as described in Methods, and have been named according to the region(s) of the HCV polyprotein which they express. A ‘prime’ indicates that the region has been truncated at either the N or C terminus, and ‘S → A’ that the active site serine (amino acid 1165) of the NS3 protease has been mutated to alanine (Fig. 1).

The recombinant virus Ac.NS3' encodes amino acids 1007–1564 of the HCV polyprotein, including the C-terminal 20 amino acids of NS2 and the conserved domain of NS3 associated with serine protease activity, but lacking the C-terminal 94 amino acids of NS3. In the virus Ac.NS3'/4', the above sequence was extended at the C terminus to include NS4A and a part of NS4B up to amino acid 1880. For each of these viruses, a counterpart carrying the Ser-1165 → Ala mutation was also produced. A further recombinant virus, Ac.NS4A, was designed to express the NS4A region: a methionine residue having been added to the N terminus.

Activity of the baculovirus-expressed NS3 protease in trans

In order to determine whether other cleavage sites within the HCV non-structural region could be processed by the NS3 protease in co-infected cells. Two further recombinant viruses were therefore produced: Ac.NS5A'/5B', which encodes amino acids 2012–2613 of the HCV polyprotein, including the NS5A/5B junction, and Ac.NS3'/4'/5', which encodes amino acids 1463–2235 including the NS3/4A, NS4A/4B, and NS4B/5A junctions (Fig. 1). These recombinant viruses were used to infect insect cells either alone or in combination with one of the NS3-expressing recombinant viruses. The expression products were analysed by Western blotting (Fig. 3).

When expressed alone, and detected using antiserum to NS5B, the NS5A'/5B' substrate appeared as a single major species of approximately 70 kDa. A range of faster-migrating immunoreactive species could also be detected, presumably resulting from non-specific proteolysis by host cell enzymes (Fig. 3 A, lane f). When co-expressed with active NS3 protease (Fig. 3, lanes f + a...
Fig. 2. Expression of NS3 proteinase and NS4A by recombinant baculoviruses. Sf9 cells were infected with recombinant baculoviruses Ac.NS3' (a), Ac.NS3'S → A (b), Ac.NS3/4' (c), Ac.NS3/4'S → A (d) and Ac.NS4A (e) as indicated. Cell extracts were analysed by Western blotting using antisera to NS3 (panel A) and NS4A (panels B and C). Cells infected with the parental wild-type AcNPV are indicated 'wt'.
Fig. 3. Cleavage of baculovirus-expressed HCV substrates in co-infected cells. Sf9 cells were infected with the recombinant baculoviruses Ac.NS5A'/5B' (f), Ac.NS3'/4'/5' (g), Ac.NS3' (a), Ac.NS3'S→A (b), Ac.NS3/4' (c) and Ac.NS3/4'S→A (d) as indicated. Cell extracts were analysed by Western blotting using antisera to NS5B (panel A), NS5A (panel D), NS3/4 (panel C) and NS4A (panels B and E). Cells infected with the parental wild-type AcNPV are indicated ‘wt’. In order to maximize detection of NS4A, the blot shown in panel E was developed using a biotinylated secondary antibody followed by streptavidin/alkaline phosphatase.

and f+c) a proportion of the substrate was cleaved to yield a product of approximately 20 kDa, the expected size for the NS5B' moiety of the substrate. This processing was not seen when Ac.NS3'S→A was used to co-infect the cells (Fig. 3, lane f+b). When antiserum to NS5A was used for detection, a 45 kDa cleaved product appeared in the presence of the active proteinase (unpublished results). These results imply that the NS5A/B junction undergoes authentic trans-cleavage in the presence of the NS3 proteinase.

Expression of the NS3'/4'/5' substrate was analysed using antisera against NS4A (Fig. 3 B), NS5A (Fig. 3 D)
and NS3/4 (Fig. 3 C). With all these antisera the unprocessed precursor was seen to migrate as a doublet, presumably due to limited proteolysis by a host cell enzyme. Upon co-infection with Ac.NS3' or Ac.NS3/4' (Fig. 3, lanes g+a and g+c), most of the precursor was processed to give three distinct products; two NS5A-immunoreactive species of approximately 30 kDa and 60 kDa (Fig. 3 D), and a 30 kDa species reacting with antiserum to NS4A (Fig. 3 B, C). Of the NS5A-immunoreactive products, the smaller is of an appropriate size to be the N-terminal moiety of NS5A, and the latter to be an NS4B-5A' partial cleavage product. The relatively low level of the latter product is consistent with this interpretation. However, this species does not react with the NS3/4 antiserum (Fig. 3 C) which has been shown to immunoprecipitate NS4B (Bartenschlager et al., 1993). It may be that the antiserum is relatively non-reactive to the N terminus of NS3B in immunoblots.

The 30 kDa cleavage product shown in Fig. 3 (B, C) is of an appropriate size to be either an NS3'-4A partial cleavage product or an NS4A-4B' partial product: we were unable to distinguish between these possibilities with the available antibodies. However, the former possibility is much the more likely, since the inefficiency of trans-cleavage at the NS3/4A junction has been shown in Fig. 2, above, and documented in the literature (Bartenschlager et al., 1994; Tomei et al., 1993; Lin et al., 1994). Although these authors were unable to detect mature NS4A being produced by trans-cleavage of an HCV polyprotein containing the whole of the NS3 domain, this may reflect the low sensitivity of their expression and detection systems. We co-infected cells with Ac.NS3' and either Ac.NS3'/4'S→A or Ac.NS3'/4'/S' and looked for the production of mature NS4A by Western blotting (Fig. 3 E). A sample of cells infected with Ac.NS3/4' was run as a control (Fig. 3, lane c) and a clear 6 kDa product was detected, representing authentic NS4A released by self-cleavage of the NS3/4A/4B' precursor. A product of identical size was produced from both the NS3/4A/4B/S→A substrate and the NS3'/4A/4B/5A' substrate, albeit at low levels (Fig. 3, lanes d+a and g+a). When co-infection was performed using Ac.NS3'S→A, no such product was detected (Fig. 3, lanes d+b and g+b). The results shown in Fig. 3 therefore demonstrate that the baculovirus-expressed proteinase is capable of trans-processing of the NS3/4A, 4A/4B, 4B/5A and 5A/5B cleavage sites in co-infected insect cells.

Lin et al. (1994) have shown that a vaccinia virus-expressed polyprotein lacking the first 166 amino acids of NS3 may undergo trans-cleavage at the NS3/4A junction, whereas no such cleavage was seen using a precursor carrying the whole of NS3 with an inactivating mutation. In contrast, we have shown low-level cleavage of the complete precursor, perhaps due to the higher expression levels in insect cells, but saw no significant increase in processing when 435 amino acids were removed from the N terminus of NS3. This suggests that some part of the NS3 region between amino acids 1193 and 1462, which was present in the truncated substrate in the study by Lin et al. (1994) but was absent from ours, may assist in the correct folding of the NS3/4A junction. Alternatively, the heterologous sequences present at the N terminus of our substrate may interfere with trans-processing.

Role of NS4A in intracellular processing

NS4A has been reported to act as a co-factor of NS3 proteinase activity: it is absolutely required for processing at the NS4B/5A junction, while cleavage at the NS5A/5B junction is enhanced by NS4A but can still take place in its absence (Bartenschlager et al., 1994; Failla et al., 1994; Lin et al., 1994). A pulse-chase experiment was carried out to determine the relative efficiency of processing of the NS5A'/B' precursor in cells co-infected with Ac.NS3', Ac.NS3/4' or a mixture of Ac.NS3' plus Ac.NS4A. Products were analysed by immunoprecipitation with anti-NS5B (Fig. 4 A). The expected increase in product with increasing chase period could not be observed, as the material degraded — after a 30 min chase, the total radio labelled protein was drastically reduced. However, an estimate of the relative processing rates was obtained by comparing levels of precursor and product immediately following the labelling period, using a phosphorimager to estimate levels of radioactivity. The results showed that in cells co-infected with Ac.NS3/4' (Fig. 4, lane f+c) or Ac.NS3' plus Ac.NS4A (Fig. 4, lane f+a+e), the NS5B' product represented approximately 40% of the total immunoprecipitated radiolabel, whereas in cells co-infected with Ac.NS3' (Fig. 4, lane f+a), NS5B' constituted less than 10% of the total. In case this reduced efficiency of processing by NS3' might result from a lower expression level of the NS3 proteinase, the pulse-chase samples were also analysed by immunoprecipitation with anti-NS3 (Fig. 4 B). The proteinase expressed by Ac.NS3/4' appeared to be more stable than that expressed by Ac.NS3', but was present at a lower level. This stability may result from the former species having the authentic NS3 C terminus. Co-infection with Ac.NS4A did not affect the stability of the Ac.NS3' expression product. [Tanj et al. (1995) reported stabilization of NS3 by NS4A in a COS-1 cell expression system, but this effect was only apparent after a chase period of 180 min or more.] Our results are consistent with the observation of Failla et al. (1994), that NS4A enhances NS3-dependent processing of the NS5A/5B junction, whether supplied in cis or in trans.
Fig. 4. Pulse-chase analysis of HCV substrate processing in co-infected cells. Sf9 cells were infected with combinations of Ac.NS5A'/B' (f), Ac.NS3/4/5' (g), Ac.NS3' (a), Ac.NS3'/4' (c) and Ac.NS4A (e) as indicated. At 24 h post-infection the cells were pulse labelled with [35S]methionine followed by a chase of 30 min to 3 h as indicated. Samples were analysed by immunoprecipitation with antisera to NS5B (panel A), NS3 (panel B) or NS5A (panel C).

A similar pulse-chase experiment was carried out to determine the relative efficiencies of processing of the NS3'-4-5' precursor by the proteinases expressed by Ac.NS3', Ac.NS3/4' and Ac.NS3' plus Ac.NS4A (Fig. 4 C). The products were analysed by immunoprecipitation using antiserum to NS5A, which reacted with three radiolabelled species: the NS3'-4-5' precursor, its NS5A' product and an NS4B/5A' intermediate cleavage product. In the cells co-infected with Ac.NS3', a gradual decrease in the level of the precursor took place over the chase period, with a concomitant increase in the level of the 5A' product. However, the level of the 4B/5A' intermediate remained comparable to that of the 5A' product, even after a 3 h chase period. The limited amount of 4B/5A' processing which has taken place must result from the trans-activation of NS3 by the NS4A supplied by the substrate molecule. When the cells were co-infected with Ac.NS3/4', the rate of disappearance of the NS3'/4-5A' precursor was similar to that observed with Ac.NS3', but the level of the NS5A' product increased at the expense of the 4B/5A' intermediate. Hence, provision of NS4A expressed in cis with the NS3 proteinase enhances the efficiency of processing at the NS4B/5A junction. In the cells co-infected with Ac.NS3' plus Ac.NS4A, it was observed that the processing of the substrate followed the pattern seen in cells co-infected with Ac.NS3' alone, even though the level of expressed NS4A was similar to that of 'released' NS4A in the Ac.NS3/4'-infected cells (unpublished results). This result contrasts with that seen for the NS5A'/5B' substrate, where NS3 was activated to a similar extent whether NS4A was supplied in cis, or as a separate
entity. Possibly some part of the NS3'/4/5' substrate interacts with NS3' in such a way as to limit transactivation by NS4A. These observations have been reproduced in a second independent experiment, and confirmed by phosphorimager analysis of products.

**In vitro activity of baculovirus-expressed NS3 proteinase**

Bouffard et al. (1995) have developed an *in vitro* assay for the NS3 proteinase based on the vaccinia virus expression system. Lysates of cells expressing either the proteinase or its substrate were mixed, and processing was shown to take place at the NS5A/B junction, but not at the NS3/4A, NS4A/4B or NS4B/5A junctions. Processing was only observed in the presence of NS4A.

We have carried out similar experiments using lysates of recombinant baculovirus-infected insect cells, and have demonstrated NS3-dependent cleavage of the NS5A'/5B substrate, but found that non-specific degradation of the expressed substrates made analysis of processing at other sites difficult (unpublished results). We have therefore developed an alternative method using *in vitro* translated substrates. During the process of construction of recombinant baculoviruses, the substrate sequences NS3'/4'/5' and NS5A'/B' were both cloned into the vector pRSET B to provide a translational start site. Coincidentally, this vector carries an upstream T7 RNA polymerase recognition site, so that the plasmids could be used to programme a coupled transcription/translation reaction, thus generating radiolabelled substrates equivalent to those expressed by the recombinant baculoviruses Ac.NS3'/4'/5' and Ac.NS5A'/5B'.

Fig. 5 (A) shows a time-course of incubation of the radiolabelled substrate NS5A'/5B' with a lysate of Ac.NS3/4'-infected cells. A gradual decrease in the level of the precursor was observed, accompanied by the appearance of two new radiolabelled species – a diffuse band corresponding in mobility to the NS5A' product, and a smaller species corresponding to NS5B'. The processing showed better time-dependence than was observed in the mixed lysate reactions, where a small proportion of the substrate was cleaved within a few minutes of mixing, and the remainder was refractory to cleavage even after extended incubation, possibly because it had undergone aggregation or association with membranes. When incubation was performed with a lysate of Ac.NS3/4'S → A-infected cells, the two products were not detected, and the level of the precursor remained unchanged up to 120 min, declining slightly thereafter.

To determine whether NS4A was required for cleavage, reactions were set up using lysates of cells infected with a series of baculoviruses (Fig. 6 A). In this experiment, more non-specific degradation of the substrate was seen in the absence of active proteinase (Fig. 6, lanes wt and d+e), but the NS5B' product was still readily visible after incubation with the Ac.NS3/4'-infected cell lysate. No evidence of specific processing was obtained with the Ac.NS3'-infected cell lysate (Fig. 6, lane a), but processing was restored when the cells were co-infected with Ac.NS3' and Ac.NS4A (Fig. 6, lane a + e 'co-inf.'), showing that NS4A is necessary and sufficient for the activation of NS3'. Finally, an incubation was performed using a mixture of lysates expressing NS3' and NS4A (Fig. 6, lane a + e 'mix'), but no evidence of processing was seen. This result suggested that NS3 and NS4A may need to associate at an early stage of expression to give rise to an active proteinase.

The failure of NS3 alone to process the NS5A/B junction was not a result of low level expression or
**Fig. 6.** Panel A. *In vitro* processing of NS5A'/B' substrate. A radiolabelled substrate (produced by *in vitro* transcription and translation of pRSET 2012-2613) was incubated for the indicated times with lysates of Sf9 cells infected with wild-type AcNPV (wt), Ac.NS3/4' (c), Ac.NS3/4'S→A (d), Ac.NS3' (a), Ac.NS4A (e), a mixture of lysates a and e (a+e mix) and a lysate of cells co-infected with Ac.NS3' and Ac.NS4A (a+e co-inf.). Samples were analysed by SDS–PAGE and fluorography. Panels B, C and D. Co-immunoprecipitation of NS3 and NS4A. Sf9 cells were infected with wild-type AcNPV (wt), Ac.NS3/4' (c), Ac.NS3' (a) and Ac.NS4A (e), or co-infected with Ac.NS3' and Ac.NS4A (a+e co-inf.). At 24 h post-infection, cells were radiolabelled with [35S]methionine. Lysates of the cells, plus a mixture of lysates a and e (a+e mix) were analysed by Western blotting with antiserum to NS4A (panel D) and also immunoprecipitated with antiserum to NS3. The immunoprecipitated material was analysed by SDS–PAGE and fluorography (panel B) and by Western blotting with antiserum to NS4A (panel C). 4A* denotes the larger form of NS4A expressed by Ac.NS4A.

Instability of the proteinase, since it was demonstrated by Western blotting that the reaction mixtures shown in lanes a and a+e 'mix' contained at least as much NS3-immunoreactive material as the mixtures shown in lanes c and a+e 'co-inf.', and there was no evidence of degradation of the proteinase during the incubation period. Similarly, NS4A was found to be stable over the incubation period whether expressed alone (Fig. 6, lane e) or *in trans* with NS3 (a+e 'co-inf.') or mixed with NS3 at the cell lysate stage (a+e 'mix') (unpublished results).

To look for any association between NS3 and NS4A, infected cells were radiolabelled with [35S]methionine, lysed and immunoprecipitated with antiserum specific for NS3. The immunoprecipitated material was analysed by SDS–PAGE and fluorography to detect NS3-immunoreactive material (Fig. 6 B) and by Western blotting with antiserum to NS4A (Fig. 6 D). Using Western blotting rather than fluorography to detect NS4A had the advantage of extra sensitivity, and also enabled the NS4A to be identified unequivocally. A small amount of NS4A was non-specifically immunoprecipitated by the NS3 antiserum (Fig. 6, lane e), but a larger amount of NS4A was immunoprecipitated from the lysate of cells co-infected with Ac.NS3 and Ac.NS4A (Fig. 6, lane a+e 'co-inf.') even though the original lysate of these cells contained a lower concentration of NS4A than the Ac.NS4A singly-infected cell lysate (Fig. 6 D). These results imply that NS4A associates with NS3, either directly or possibly via a cellular component. When cell lysates containing NS3 and NS4A were mixed prior to immunoprecipitation (Fig. 6, lane a+e 'mix') a very small amount of NS4A was detected - this is probably due to non-specific immunoprecipitation. The apparent lack of association between NS3 and NS4A may account for the failure of such a lysate to process the NS5A'/B' substrate *in vitro*. It may result from the low concentrations of the two proteins in comparison to a co-infected cell, or from one or both proteins having become aggregated or misfolded when expressed alone. Co-immunoprecipitation of NS3 and NS4A has also
been reported by Failla et al. (1995), although under less denaturing conditions than we have used.

The lysate of cells infected with Ac.NS3/4′ contained substantially less NS3 than the Ac.NS3′ + Ac.NS4A co-infected cell lysate (Fig. 6 B), but was more efficient in processing the in vitro translated substrate (Fig. 6 B). This might result from a proportionately larger amount of the NS3 having NS4A associated with it (Fig. 6 C, lane c) or from the C-terminal 94 amino acids of NS3, which are missing from the Ac.NS3′-expressed protein, being necessary for the enzyme to be fully active.

Fig. 7 (A) shows the in vitro processing of an in vitro translated NS3′/4′/5′ substrate by baculovirus-expressed proteinase. As with the NS5A′/B′ substrate, no processing was observed in the presence of NS3′ alone (Fig. 7, lane a). The expression product of Ac.NS3′/4′, however, caused the substrate to be cleaved to two smaller products (Fig. 7, lane c). The size of the larger species was consistent with it being an NS4B/5A′ partial cleavage product, as observed in co-infected insect cells (Figs 3 D and 4 C), while the smaller product was shown to co-migrate with an NS3′/4A partial product (Fig. 3 C). These assignments were supported by immunoprecipitation with antiserum to NS3′/4, which is known to react with NS3, NS4A and NS4B, and antiserum to NS5A. Anti-NS3′/4 immunoprecipitated both digestion products, whereas anti-NS5A immunoprecipitated the larger product only (Fig. 7 B). No mature NS3 or NS5A′ could be detected. We concluded, therefore, that the radiolabelled NS3′/4′/5′ substrate can be processed in vitro at the NS4A/4B junction, but not at the NS3′/4A or NS4B/5A junctions. These results are in accord with those obtained by co-infection of cells, where we have shown that the NS3′/4A and NS4B/5A junctions are less susceptible to trans-cleavage than the NS4A/4B junction. As with the NS5A/5B junction, processing took place only in the presence of NS4A, and was prevented by the mutation of Ser-1165 to Ala in NS3. Our results differ from those of Bouffard et al. (1995) who could demonstrate in vitro processing at the NS5A/5B junction but not at the NS4A/4B junction. This discrepancy may result from differences in sensitivity of our respective assays, or from the substrate molecules assuming different conformations when expressed in different systems. An absolute requirement for NS4A for in vitro proteinase activity was also observed by Bouffard et al. (1995), and contrasts with the situation in co-infected cells. One possible explanation for this difference is that a host cell factor may substitute to some extent for NS4A in vivo but becomes dissociated from NS3 in the relatively dilute in vitro reaction. This dilution effect might also cause NS4A to become essential in vitro if its role were to bring proteinase and substrate together by interacting with both. Further studies will be necessary to test these hypotheses.

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