Biosynthesis of Classical Swine Fever Virus Nonstructural Proteins[∇]

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Proteolytic processing of polyproteins is considered a crucial step in the life cycle of most positive-strand RNA viruses. An enhancement of NS2-3 processing has been described as a major difference between the noncytopathogenic (non-CP) and the cytopathogenic (CP) biotypes of pestiviruses. The effects of accelerated versus delayed NS2-3 processing on the maturation of the other nonstructural proteins (NSP) have never been compared. In this study, we analyzed the proteolytic processing of NSP in Classical swine fever virus (CSFV). Key to the investigation was a panel of newly developed monoclonal antibodies (MAbs) that facilitated monitoring of all nonstructural proteins involved in virus replication (NS2, NS3, NS4A, NS5A, and NS5B). Applying these MAbs in Western blotting and radioimmunoprecipitation allowed an unambiguous identification of the mature proteins and precursors in non-CP CSFV-infected cells. Furthermore, the kinetics of processing were determined by pulse-chase analyses for non-CP CSFV, CP CSFV, and a CP CSFV replicon. A slow but constant processing of NS4A/B-5A/B occurred in non-CP CSFV-infected cells, leading to balanced low-level concentrations of mature NSP. In contrast, the turnover of the polyprotein precursors was three times faster in CP CSFV-infected cells and in cells transfected with a CP CSFV replicon, causing a substantial increase of mature NSP concentrations. We conclude that a delayed processing not only of NS3 but further of all NSP represents a hallmark of regulation in non-CP pestiviruses.

Classical swine fever virus (CSFV) belongs to the genus *Pestivirus*, which also comprises *Bovine viral diarrhea virus* (BVDV) and *Border disease virus* (BDV). Pestiviruses, together with flaviviruses and hepatitis C virus, form the family *Flaviviridae* (31).

The single-stranded RNA of CSFV has a size of 12.3 kb and encodes a single hypothetical polyprotein of 3,899 amino acids which is further processed into 12 mature proteins by cellular and viral proteases (16, 23). Studies of CSFV- and BVDV-derived defective interfering particles have shown that NS3, NS4A, NS4B, NS5A, and NS5B are sufficient for genome replication and thus encompass all necessary enzymatic functions (5, 18).

Noncytopathogenic (non-CP) and cytopathogenic (CP) pestivirus strains were distinguished according to the induction of apoptosis in infected cultured cells. Acute infections with non-CP BVDV and non-CP BDV usually result in mild clinical symptoms in ruminants but can induce abortion or persistent infection of the offspring, depending on the stage of gestation. In contrast, classical swine fever (CSF) is a hemorrhagic fever-like disease that causes disastrous epidemics in pigs. Infections with non-CP CSFV strains are causative for the development of this disease. Depending on the clinical manifestation of CSF, high-, moderate-, and low-virulence strains have been identified.

Cytopathogenic pestiviruses occur sporadically in nature and are characterized by mutations that allow accelerated replication. CP BVDV isolates emerge by nonhomologous RNA re-

combination in animals persistently infected with the parental non-CP BVDV strain. The appearance of CP BVDV strains in persistently infected cattle induces the lethal mucosal disease (3, 4). However, acute infections with these CP BVDV strains cause no severe disease in naïve hosts and do not induce persistent infections of the offspring during gestation. By comparison, CP CSFV isolates were rarely identified in the field and always represented helper virus-dependent defective interfering particles (DI) (19). A laboratory CP CSFV strain has been constructed (termed CP CSFV-JIV) according to the genomic organization of the cytopathic BVDV strain CP8 (10). The CP CSFV-JIV was highly attenuated in pigs compared to the parental non-CP CSFV (10).

Molecular analyses have shown that genomic changes of CP pestiviruses induce a continuous expression of high levels of mature NS3, while its precursor (NS2-3) is dominant in non-CP pestiviruses. Since replication in CP pestiviruses is very efficient, mature NS3 is considered to be the central regulator of viral replication (15). Current knowledge about pestivirus NSP is mainly based on the analysis of cytopathogenic strains of BVDV. For CP BVDV the NSP have been mapped using antisera against bacterial fusion proteins (6). Furthermore, precursor-product relationships were demonstrated and the mature viral proteins were specified in tissue culture cells infected with CP BVDV (7, 17). The precise cleavage sites were determined for CP BVDV in recombinant systems by radiosequencing (28, 34).

The serine protease NS3 and its cofactor NS4A mediate the generation of mature NSP in CP BVDV by four cleavages (NS3/NS4A, NS4A/NS4B, NS4B/NS5A, and NS5A/NS5B) at Leu/Ser or Leu/Ala sites. NS2, NS3, and NS2-3 have been identified as p54, p80, and p125. Mature NS4A and two NS4A-containing precursor molecules were described as p10, p42, and p175, re-

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FIG. 1. Genome organizations of the parental non-CP CSFV clone (center) and its derivatives, CP CSFV replicon (top) and CP CSFV-JIV (bottom). Relative positions of viral genes encoding structural proteins (Core, E^{ns}, E1, and E2) and nonstructural proteins (N^{pro}, P7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are indicated. The CP CSFV replicon lacks the coding region for Core to NS2. Substitution of Val₂₂₉₉ by a methionine residue allowed metabolic labeling of NS4A with [³⁵S]Met in the modified CP CSFV replicon V2299M. CP CSFV-JIV contains an insertion of 1.539 kb derived from the CP BVDV strain CP8 (GenBank accession number AY182137.1). The inserted sequence encodes a complex fusion protein (513 amino acids) that is composed of viral (Core*, E^{ns}*, and N^{pro}*) as well as cellular (JIV-1*, JIV-2*, and bovine homologue to human nuclear protein 1, Hcc-1*) sequences.

spectively (7). NS4B was detected as p30 for the CP BVDV strains Pe515cp and CP7 (17, 29) and as p38 for the CP BVDV strain NADL (32). BVDV NS5A was demonstrated as p58 and as part of the precursors p96, p133, p165, and p175 (1, 7). The three precursors p133, p165, and p175 were assigned to the viral polymerase NS5B together with the mature form p75. Since the cleavage sites are highly conserved among all pestiviruses, these proteins served as a reference for the genomic organization of other pestiviruses, including CSFV (28).

Analyses of NS4-5 maturation in non-CP pestiviruses have never been put forward, probably because of generally lower expression levels and the lack of proper serological reagents. Since the proteolytic maturation of the NSP is indispensable for viral replication, we hypothesized that a balanced maturation of NS4-5 precursors represents a key element of regulation in non-CP pestiviruses. Here we report on the identification of the nonstructural proteins in CSFV-infected cells and compare the kinetics of nonstructural protein maturation between non-CP CSFV, CP CSFV-JIV, and a CP CSFV replicon.

MATERIALS AND METHODS

Cells and viruses. SK-6 cells (12) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). These cells were maintained at 37°C and 5% CO₂.

Non-CP CSFV was generated by transfection of a SP6 transcript of the infectious cDNA clone p447 (based on strain Alfort-Tübingen) in SK-6 cells as

described previously (22). Nucleotide and amino acid numbers of CSFV throughout this study refer to the sequence of this strain (GenBank J04358.2). The genetically engineered CP CSFV-JIV was generated by transfection of a SP6 transcript of the infectious cDNA clone p447-JIV as described previously (10). A CP CSFV replicon was constructed in analogy to the genomic organization of the BVDV defective interfering particle 9 (GenBank U03912.1; 27). The SP6 transcript of the cDNA clone p447-rep was transcribed by electroporation as well. The genetic organization of non-CP CSFV, CP CSFV-JIV, and the CP CSFV replicon is presented in Fig. 1.

CSFV infection and metabolic labeling. SK-6 cells were infected with a multiplicity of infection (MOI) of 1 for Western blot analyses and with an MOI of 10 for metabolic labeling. Protein labeling and transfection of RNA were done essentially as described before (27). Briefly 1×10^6 non-CP CSFV, CP CSFV-JIV-infected, CP CSFV replicon-transfected, or uninfected SK-6 cells were washed with phosphate-buffered saline (PBS) and incubated with FCS-deficient DMEM without cysteine and methionine at 37°C for 1 h. Cells were labeled with 250 μ Ci of a mixture of [³⁵S]methionine-cysteine (Hartmann Analytik, Braunschweig, Germany) in DMEM without cysteine and methionine for 1 h. The labeling medium was then removed, and cells were washed three times with PBS and harvested in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.5% Na-deoxycholate, 1% NP-40, 0.5 mM PefaBloc SC [Roche], pH 8.0) with 2% SDS. For chase experiments, DMEM with 1 mM unlabeled methionine and cysteine was added after washing and cells were harvested after the indicated time.

Generation of bacterial expression plasmids. For expression in *Escherichia coli* the coding sequences of the NSP were ligated into a modified pet11a vector (Novagen) with an N-terminal heparithistidine tag (MASHHHHHHH). The resulting plasmids were named p1039 (NS3), p1144 (NS4A), p1117 (NS4B), p1142 (NS5A), and p1141 (NS5B). For construction of GST-NS2-3p (pL68) the vector pGex-6P (Amersham) was used. In the case of pL68 a polyhistidine tag and a stop codon were inserted behind an existent XhoI site. Mutagenesis was per-

TABLE 1. Primers used to amplify defined fragments of the CSFV genome

Primer	Sequence
PR1	5'-AACTCGAGATGACTGGTGGACAGCAAATGGGT-3'
PR2	5'-ATGATGATGATGATGATGATGGCTAGCCATATG-3'
PR3	5'-ACAGCAGAGAACGCCCTGCTAGTCGC-3'
PR4	5'-AACTCGAGCTACTTCAATTCAGTTTCCTTC-3'
PR5	5'-CCAGGAGACACAGAAACACAAC-3'
PR6	5'-AACTCGAGTAGCTGGCGAATTTTCCCTC-3'
PR7	5'-TCGAGCAATTACATACTAGAGC-3'
PR8	5'-AACTCGAGCTACATAGAATACACTTTCGCGC-3'
PR9	5'-AGTAACTGGGTGATACAAGAAG-3'
PR10	5'-AACTCGAGTCACACCCCTTCTATCAGG-3'
PR11	5'-TTTGGATCCTTGCTGCTAGTAGGGTCAAAGAG-3'
PR12	5'-AACTCGAGTTAGAGCTTGGTTGGTTTGAATC-3'

formed by PCR with *Pfu* DNA polymerase (Promega). All mutagenized DNA plasmids were verified by nucleotide sequencing.

The pet11a vector was amplified by PCR with an antisense polyhistidine primer (PR1) and a sense primer with an additional XhoI site (PR2). The sequences of NS4A (amino acids [aa] 2273 to 2336, PR3 and PR4, p1144), the C-terminal part of NS4B (aa 2461 to 2683, PR5 and PR6, pL117), NS5A (aa 2684 to 3180, PR7 and PR8, p1142), and NS5B (aa 3181 to 3898, PR9 and PR10, p1141) were amplified by PCR as well. After digestion with XhoI, vector and insert were ligated with T4-DNA ligase (BioLabs). A polypeptide fragment consisting of the C-terminal part of NS2 and the N-terminal part of NS3 (aa 1435 to 1780, PR11 and PR12, pL68) was amplified by PCR. The modified pGex-6P vector and this insert were digested with BamHI and XhoI and ligated. Primer sequences are indicated in Table 1.

Preparation of recombinant proteins. The heterologous expression of the CSFV proteins NS2 to NS5B was optimized using different approaches. In most cases the expression of N-terminally heptahistidine-tagged proteins via a T7 RNA polymerase promoter containing plasmid (pET11a) in *E. coli* strain Rosetta 2 (Novagen) succeeded. Expression of the recombinant proteins was performed at 30°C with vigorous aeration for 2 h in transformed cells that were grown in Luria-Bertani medium at 37°C and induced at an optical density (OD) of 0.8 with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; AppliChem). Cells were harvested by centrifugation, resuspended in 50 mM Na₂PO₄, 300 mM NaCl, pH 7.0 to 8.0, and lysed by three cycles of freezing and thawing. Insoluble matter was removed by ultracentrifugation at 10⁵ × g for 1 h. Quantities of about 0.5 mg of each of the soluble protein species were obtained from 250-ml cultures. In the case of p1142 and pL68, urea was added to a final concentration of 8 mol/liter after cell lysis. Only the purification of NS4B (pL117) was performed in the presence of 4 mol/liter guanidinium chloride. All proteins were purified by ion metal affinity chromatography (IMAC) with Ni²⁺ Sepharose (HisTrap; GE Healthcare) as recommended by the supplier. The amount and purity of proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and identity was confirmed by immunoblot analysis with an anti-His antibody. Purified proteins were dialyzed against PBS and used for immunization of mice or served as an antigen source for the enzyme-linked immunosorbent assay (ELISA) screening. A map of all expression constructs is given in Fig. 2.

Generation of monoclonal antibodies (MAbs). Hybridomas secreting the desired antibodies against the different NSP of CSFV were generated according to the standard procedure (13, 26). Briefly, female BALB/c mice were immunized intraperitoneally with 20 µg of purified proteins emulsified in Freund's incomplete adjuvant (Sigma) subsequently at 3-week intervals. Serum samples (0.1 ml/mouse) were obtained prior to the first immunization and a week after each booster immunization. The animals' response to immunogen was assessed by immunoblot analysis with recombinant proteins. After seroconversion, mice were finally boosted with 20 µg protein on the following 3 days without adjuvant. On the fourth day, the mouse spleens were dissected, and cells were prepared and fused with sp2/0-AG14 myeloma cells. Fusion was induced with 50% (vol/vol) polyethylene glycol (molecular weight, 1,500; Roche) for 1 min at 37°C. Afterwards the cells were grown in DMEM supplemented with 20% F10 (GIBCO), 20% F12 (GIBCO), 15% FCS, 2 mM L-glutamine, oxaloacetate-pyruvate-insulin supplement (OPI; Sigma), and hypoxanthine-aminopterin-thymidine supplement (HAT; Sigma) and seeded on 96-well cell dishes.

Characterization of generated hybridoma cultures. ELISA screening of hybridoma cell culture supernatant was performed after fusion from day 7 to 10

FIG. 2. Location of the bacterial expression constructs within the CSFV polyprotein. Bars symbolize CSFV nonstructural proteins 2, 3, 4A, 4B, 5A, and 5B. Individual domains of NS3 are depicted (3p, NS3 protease domain; 3h, NS3 helicase domain). Names of the particular expression plasmids are indicated below the bars. Numbers in parentheses indicate the amino acid (AA) positions in the CSFV polyprotein (GenBank accession number AAA43844.2). Asterisks indicate truncated gene products. The positions of glutathione-S-transferase (GST) and the polyhistidine tag (His₇) are shown for each construct.

according to standard protocols (9). Recombinant proteins were dissolved in ELISA coating buffer (0.1 sodium carbonate, pH 9.5) and diluted to a final concentration of 0.2 µg/ml. ELISA 96-well plates (Maxisorb; Nunc) were coated and blocked with 10% FCS. For coating, insoluble proteins that had been solubilized in 8 M urea were directly diluted in the coating buffer without prior dialysis. After incubation with hybridoma cell culture supernatant, specific MAbs were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (Dianova) and 3,3',5,5'-tetramethylbenzidine (TMB; Sigma). Hybridomas from wells with ELISA OD values above 0.7 were further selected by serial limited dilution and rescreened until stable antibody-producing cell lines remained. To verify antibody specificity, immunoblot analyses with recombinant proteins were performed. Glutathione-S-transferase (GST) with a C-terminal polyhistidine tag was expressed from the modified pGEX-6P vector without the NS2-3 fusion peptide insert. Complete NS3 was expressed with an N-terminal polyhistidine tag from plasmid p1039. A differentiating immunoblot screening using GST, NS3, and the GST-NS2-3p fusion protein allowed for isolation of hybridomas directed against NS2 as well as against the NS3 protease domain (NS3p). Furthermore, IgG subtypes and the reactivity against authentic pestivirus proteins were determined for selected antibodies. The nomenclature of hybridomas and properties of secreted MAbs used in this study are indicated in Table 2.

TABLE 2. Nomenclature of selected hybridomas and properties of the secreted MAbs

MAb (IgG subtype)	Specificity	Properties ^a	Cross-reactivity with BVDV strain NCP7/890 ^b
GL21 (IgG1)	NS2	WB, IF, IP	Y/Y
GL22 (IgG1)	NS2	WB, IF, IP	Y/Y
GL3p1 (IgG1)	NS3	WB, IP	Y/Y
GL3p2 (IgG1)	NS3	WB, IP	Y/Y
GH4A1 (IgG2b)	NS4A	WB, IF, IP	Y/Y
GH4A2 (IgG2b)	NS4A	WB, IF, IP	Y/Y
GL4B1 (IgG1)	NS4B	IP	Not done
GL4B2 (IgG1)	NS4B	IP	Not done
GL5A1 (IgG1)	NS5A	WB, IF, IP	N/N
GL5A2 (IgG1)	NS5A	WB, IF, IP	N/N
GR5B1 (IgG1)	NS5B	WB, IF, IP	N/N
GR5B2 (IgG2b)	NS5B	WB, IP	N/N

^a WB, reactivity in immunoblotting; IF, immunofluorescence; IP, immunoprecipitation.

^b Y, reactive; N, not reactive.

SDS-PAGE and immunoblotting. Proteins were separated in polyacrylamide-tris gel systems (24). After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Pall Corporation). The membrane was blocked with 4% dried skim milk (wt/vol) in PBS with 0.1% Tween 20 (vol/vol). After incubation with primary antibodies or sera, HRP-conjugated goat anti-mouse IgG and chemiluminescence reagent (Western Lightning Plus-ECL; Perkin-Elmer) were applied for signal detection.

Indirect immunofluorescence. Cells that had been infected with non-CP CSFV and CP CSFV-JIV and mock-infected control cells were seeded on 24-well cell culture dishes as described above. At 24 h after infection, cells were fixed with 4% paraformaldehyde (wt/vol) in PBS and permeabilized with 0.5% Triton X-100 (Fluka). Fixed cells were incubated with the respective hybridoma cell culture supernatants diluted 1:5 in PBS. A cyanogen-3-labeled goat anti-mouse immunoglobulin (Dianova) was added, and indirect immunofluorescence was monitored by a TCS SP5 confocal microscope (Leica).

Radioimmunoprecipitation. Cells (1.5×10^6) were lysed in 250 μ l RIPA buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [vol/vol] NP-40, 1% [wt/vol] deoxycholate, 2% [wt/vol] SDS, 0.5 mM PefablocSC [Merck]), incubated for 10 min at 95°C, and frozen. After being thawed, the lysates were clarified by centrifugation at $1,600 \times g$ for 30 min at 4°C. The supernatant was diluted in 2.25 ml RIPA buffer without SDS to a final concentration of 0.2% SDS. A 100- μ l sample of crude hybridoma supernatant containing ca. 5 μ g of antibody was added to 1.25 ml of the cell lysates, and the mixture was incubated for 1 h at 4°C. For precipitation the lysates were mixed with 100 μ l of 25% (vol/vol) protein-G-Sepharose (GE Healthcare). After an incubation period of 1 h at 4°C, the precipitates were collected by centrifugation at $3,000 \times g$ for 5 min and washed three times with RIPA buffer. Precipitates were dissolved in SDS-PAGE sample buffer, boiled for 5 min, and analyzed by SDS-PAGE. Gels were fixed with 40% methanol and 10% acetic acid. Autoradiography was enhanced for fluorography with enlightening solution (Amplify; GE Healthcare), and signals were analyzed with Kodak films.

Calculation of NSP half-lives. Quantification of radioactive signals was achieved by phosphorimaging of dried gels (Typhoon 9200; GE Lifescience). Intensity data of individual protein bands before chase (0 h) and after each chase period were fit to a first-order decay function to calculate protein turnover and stability. To combine the data from several experiments, we calculated the relative signal intensity as a percentage. The arithmetic average was calculated, and linear regression was performed. Relative counts from very late chase time points (12 h and 24 h) were not included in these analyses, because no linear correlation was seen. The gradient of the regression line was used to calculate the half-lives of individual proteins, and the resulting half-lives were rounded (see Fig. 7). For calculations of the mature protein's half-lives, we subtracted the estimated supply from precursors.

RESULTS

Identification of CSFV nonstructural proteins. Despite 25 years of active research, most of the CSFV NSP have never been demonstrated in infected cells. To provide a basis for an in-depth analysis of processing and biosynthesis, we had to identify and characterize CSFV NSP.

Our newly developed hybridomas were primarily identified by ELISA against the bacterially expressed antigens. To analyze the reactivity of MAbs against the authentic viral antigens and to compare the NSP expression, we performed indirect immunofluorescence assays with non-CP CSFV and CP CSFV-JIV-infected SK6 cells. Some of these MAbs were not useful for immunofluorescence application because of low signal intensities or unspecific reactions with uninfected SK6 cells. Both MAbs against the N terminus of NS3 and both MAbs against NS4B failed to react with viral proteins in indirect fluorescence assays. The MAbs specific for NS2 (GL21), NS4A (GH4A1), NS5A (GL5A1), and NS5B (GR5B1) clearly recognized the respective viral proteins in non-CP CSFV and CP CSFV-JIV-infected cells. For the detection of NS3, Mab 8.12.7, kindly provided by E. J. Dubovi (Cornell University, Ithaca, NY), was used (8). The immune reaction of MAbs against NS3, NS4A, NS5A, and NS5B resulted in focal fluorescence signals in the

FIG. 3. Indirect immunofluorescence analyses of mock-infected (left column), non-CP CSFV-infected (middle column), and CP CSFV-JIV-infected SK-6 (right column) cells using confocal laser microscopy. Cells were fixed 24 h after infection (MOI, 0.1) and incubated with monoclonal antibodies indicated at the left. Immune complexes were detected with a cyanogen-3-labeled goat anti-mouse immunoglobulin. The signals revealed for NS2, NS3, NS4A, NS5A, and NS5B in infected SK-6 cells show cytoplasmic localization and a distinctly perinuclear shape for NS3, NS4A, and NS5B.

cytoplasm of non-CP CSFV-infected cells (Fig. 3). In contrast, fluorescence signals of MAbs against NS3, NS4A, NS5A, and NS5B were broadly distributed through the cytoplasm in CP CSFV-JIV-infected SK-6 cells. Signal intensities were higher in the case of CP CSFV-JIV-infected cells than in non-CP CSFV-infected cells, indicating a generally higher level of protein expression for the cytopathogenic biotype of CSFV.

Extracts from SK-6 cells 48 h postinfection with non-CP CSFV were used to identify the NSP and their respective precursors with the panel of monoclonal antibodies in immunoblot analyses. Application of MAbs GL21 and GL22 against NS2 revealed a predominant reactivity with p125 (NS2-3). After extended exposure, an additional protein, with an apparent molecular mass of 54 kDa, was observed (Fig. 4A), which corresponds to the calculated molecular mass of NS2 with 456 amino acids (52 kDa). CSFV NS2 has so far not been demonstrated. MAbs GL3p1 and GL3p2 that were directed against the N-terminal portion of NS3 detected p125 (NS2-3) and additional weak signals with an apparent molecular mass of 75 kDa which correspond to NS3 (Fig. 4B). NS3 (682 aa; calculated molecular mass, 78 kDa) has been visualized earlier in non-CP CSFV-infected cells as p75/p80 (NS3) and p120/p125

FIG. 4. Immunoblot analysis of non-CP CSFV-infected (lanes 2) and mock-infected SK-6 (lanes 1) cells. At 48 h postinfection, the cells were washed with PBS and harvested. The samples were subjected to SDS-PAGE under reducing conditions, transferred to nitrocellulose membranes, and analyzed using MAbs specific for NS2 (GL21 [A]), NS3 (GL3p1 [B]), NS4A (GH4A1 [C]), NS5A (GL5A1 [D]), and NS5B (GR5B1 [E]). Molecular mass markers are indicated in kilodaltons on the left. The positions of defined nonstructural proteins and precursors are indicated at the right margin. Immunoblot analysis with MAb GH4A1 against NS4A resulted in strong background signals from cellular proteins. Additional specific signals which appeared with MAb GL5A1 against NS5A (45 kDa, 78 kDa, and 100 kDa) and with GRS5B1 against NS5B (100 kDa) were not further characterized.

(NS2-3) (11, 30). Immunoblot analysis with NS4A-specific antibodies (GH4A1-6) allowed detection of NS4A in non-CP CSFV-infected cells. A protein of 11 kDa corresponds to mature NS4A (63 aa; calculated molecular mass, 7 kDa), while signals at 42 kDa and 170 kDa represent NS4A/B and NS4-5, respectively (Fig. 4C). Our MAbs against NS4B (GL4B1, GL4B2) failed to detect specific signals in immunoblot analysis of non-CP CSFV-infected cells (data not shown). Analysis with NS5A-specific MAbs (GL5A1-7) yielded signals with apparent molecular masses of 54 kDa and 56 kDa corresponding to mature CSFV NS5A (497 aa; calculated molecular mass, 56 kDa). The double band at 54 kDa and 56 kDa is most likely due to differences in phosphorylation, which had been observed in CP BVDV as well as hepatitis C virus (HCV) NS5A

(21). Additional signals at 94 kDa (NS4B-5A), 130 kDa (NS5A-B), and 170 kDa (NS4-5) originate from polyprotein precursors (Fig. 4D). The MAbs directed against NS5B (GR5B1) led to detection of proteins with apparent molecular masses of 78 kDa, 130 kDa, and 170 kDa that correspond to mature NS5B (717 aa; calculated molecular mass, 81 kDa), NS5A/B, and NS4-5, respectively (Fig. 4E).

Determination of NSP synthesis by radioimmunoprecipitation. Immunoblot experiments have shown that all nonstructural proteins can be detected in non-CP CSFV-infected cells either as mature molecules or as part of a precursor with the exception of NS4B. Since proteins can accumulate over the entire duration of infection, immunoblotting is not appropriate for the determination of precursor-product relationships.

Thus, radioimmunoprecipitation experiments were performed to determine the synthesis products shortly after translation. SK-6 cells were infected with non-CP CSFV at an MOI of 10. Infected SK-6 cells were pulse-labeled at 24 h postinfection (p.i.) with [35 S]methionine for 1 h, washed with PBS, and lysed under denaturing conditions in RIPA buffer.

Radioimmunoprecipitation of non-CP CSFV-infected cells with the NS2-specific antibody GL21 revealed the unprocessed NS2-3 molecule with a strong signal at 125 kDa (Fig. 5A, lane 1). The MAbs GL3p1, specific for the N-terminal part of NS3, yielded a weak signal at about 75 kDa, corresponding to the mature NS3, and a stronger signal at 125 kDa (NS2-3; Fig. 5A, lane 2). The mature NS4A of CSFV could not be labeled with [35 S]methionine-cysteine, because it lacks the respective residues. After precipitation with the NS4A-specific MAb GH4A1 (Fig. 5A, lane 3) and with the NS4B-specific MAb GL4B1 (Fig. 5A, lane 4) only a weak signal at 170 kDa (NS4-5) was observed. Neither the NS4A/B precursor nor mature NS4B was visible. Precipitation experiments with the NS5A-specific MAb GL5A1 led to signals at 94 kDa (NS4B-5A), 130 kDa (NS5A/B), and 170 kDa (NS4-5; Fig. 5A, lane 5). Proteins precipitated with the NS5B-specific MAb GR5B1 yielded a very weak signal at 78 kDa, corresponding to mature NS5B, and stronger signals at 130 kDa (NS5A/B) and 170 kDa (NS4-5; Fig. 5A, lane 6).

The precipitation experiments with non-CP CSFV-infected cells indicate that the majority of NSP precursors remain unprocessed after 1 h of labeling. The NS4-5 precursor together with the processing intermediates NS5A/B and NS4B/5A lead to clear signals, while the mature NS5A and NS5B are hardly detectable.

To determine whether the observed slow generation of NSP in non-CP CSFV-infected cells is characteristic for the noncytopathogenic phenotype of pestivirus replication, biosynthesis of NSP was studied in a homologous CP CSFV replicon. This CP CSFV replicon was constructed to be analogous to a CP BVDV replicon (DI9) that lacked all structural proteins, p7, and the entire NS2 (27) (Fig. 1). This deletion generates a N^{pro}-NS3 fusion protein that, due to an efficient cleavage of the N-terminal autoprotease N^{pro}, results in the release of mature NS3 (19). As shown for related BVDV replicons, the CP CSFV replicon expressed NS3 to high levels and host cells are lysed within 36 h after transfection (data not shown). Radioimmunoprecipitation was performed with SK-6 cells 8 h after transfection of RNA. Cells were pulse-labeled with [35 S]methionine-cysteine for 1 h and washed with PBS, and the lysate was used for precipitation. For the CP CSFV replicon, the MAbs against NS4A (GH4A1) and NS4B (GL4B1) revealed a protein with an apparent molecular mass of 170 kDa (NS4-5; Fig. 5B, lane 1 and 2). An antibody specific for NS5A (GL5A1) precipitated proteins of 54 kDa (NS5A), 94 kDa (NS4B-5A), 130 kDa (NS5A/B), and 170 kDa (NS4-5; Fig. 5B, lane 3). The MAb GR5B1 against NS5B yielded signals at 78 kDa (NS5B), 130 kDa (NS5A/B), and 170 kDa (NS4-5; Fig. 5B, lane 4). In contrast to the results obtained with non-CP CSFV-infected cells, strong signals of mature NS5A and NS5B were detected for the CP CSFV replicon. These results suggest that processing of NS4-5 precursors is more rapid in the context of a CP CSFV replicon expressing mature NS3.

Differences between the transfection of subgenomic viral RNAs and the infection of cells with non-CP CSFV might also

influence NSP processing. To overcome this limitation, we included analyses of the genomic CP CSFV-JIV. The laboratory CP CSFV-JIV is a complex construct which was derived from the parental non-CP CSFV used in this study (10). CP CSFV-JIV was genetically engineered by the insertion of foreign genes from a CP BVDV isolate (BVDV CP8). The inserted sequence encodes, among other cellular and viral proteins, the cellular protein JIV, which enhances NS2-3 cleavage as shown in Fig. 1. Infection with CP CSFV-JIV induces apoptosis of the host cells within 48 h (10). Radioimmunoprecipitation was performed with SK-6 cells 24 h after infection. Cells were pulse-labeled with [35 S]methionine-cysteine for 1 h and washed with PBS, and the lysate was used for precipitation. In these lysates the antibody against NS2 (GL21) precipitated proteins of 43 kDa (a fragment of NS2) and 125 kDa (NS2-3; Fig. 5C, lane 1), while an antibody against NS3 (GL3p1) precipitates proteins of 75 kDa (NS3) and 125 kDa (NS2-3; Fig. 5C, lane 2). For CP CSFV-JIV, the MAbs against NS4A (GH4A1) and NS4B (GL4B1) detected a protein with an apparent molecular mass of 170 kDa (NS4-5; Fig. 5C, lane 3 and 4). The antibody against NS5A (GL5A1) showed proteins of 54 kDa (NS5A), 94 kDa (NS4B-5A), 130 kDa (NS5A/B), and 170 kDa (NS4-5; Fig. 5C, lane 5) and the MAb specific for NS5B (GR5B1) yielded signals at 78 kDa (NS5B), 130 kDa (NS5A/B), and 170 kDa (NS4-5; Fig. 5C, lane 6). Analyses present strong signals of mature NS5A and NS5B, suggesting that processing of NS4-5 precursors is more rapid not only for the subgenomic CP CSFV replicon but also for the genomic CP CSFV-JIV. The experiment further demonstrates that the expression pattern of a CP CSFV-JIV is clearly reproduced by the CP CSFV replicon.

Kinetics of NSP maturation. To study the kinetics of precursor processing, pulse-chase experiments were performed. SK-6 cells were infected with non-CP CSFV at an MOI of 10. Pulse-labeling was done at 24 h, 48 h, or 72 h p.i., and the chase was started by removal of the labeling medium, washing, and replenishment with medium containing excess amounts of unlabeled methionine and cysteine residues. Cells were lysed after 0, 0.5, 1.5, 4.5, and 24 h and subjected to immunoprecipitation using the panel of MAbs. For analyses of the CP CSFV replicon, RNA was transfected by electroporation and pulse-labeling was started only at 8 h posttransfection. Chase experiments were performed as described for the infection with non-CP CSFV. Pulse-labeling of CP CSFV-JIV NSP was performed at 24 h postinfection, and translation products were chased for 0, 0.5, 1.5, 4.5, and 12 h. Immunoblotting and precipitation experiments demonstrated a cotranslational cleavage between NS2-3 and NS4-5.

Because of the genomic deletion of NS2, a NS2-3 molecule is not encoded by the CP CSFV replicon (Fig. 1). Instead, a 95-kDa precursor was apparent, which completely decayed within 4.5 h of chase and most likely represented an N^{pro}-NS3 precursor molecule. The signal of mature NS3 (80 kDa) increased within the first 0.5 h of chase and remained stable for several hours (Fig. 6B). The calculated half-life of mature NS3 is about 4 h for the CP CSFV replicon (Fig. 7D). MAbs against NS2 and NS3 detected the 125-kDa precursor in non-CP CSFV-infected cells during the entire chase period. Concurrently, the mature NS3 (80 kDa) was apparent, while NS2 was not detectable (Fig. 6A and B). Quantification of NS3 signals

FIG. 5. Immunoprecipitation of pestivirus nonstructural proteins. SK-6 cells were metabolically labeled 24 h postinfection with non-CP CSFV (A), 8 h after transfection of CP CSFV replicon RNA (B), and 24 h postinfection with CP CSFV-JIV (C). The precipitated proteins were analyzed by SDS-PAGE and autoradiography. Specificity of applied MAbs is shown above the lanes. ^{14}C -labeled molecular mass markers are indicated in kilodaltons on the left. The positions of NS2-3 and NS3 are marked at the left, while proteins containing NS4 and NS5 are marked at the right margin. Note that NS4-5, and NS5A/B precursor molecules are clearly visible in non-CP CSFV-infected cells, while mature NS3 and NS5B are hardly detectable. In contrast, immunoprecipitation with cells transfected with the CP CSFV replicon and with cells infected with CP CSFV-JIV yielded strong signals of both precursor molecules and mature nonstructural proteins. Specificity of detected proteins was confirmed by precipitation experiments with mock-infected SK-6 cells (data not shown). A background band observed in precipitation experiments with MAbs against NS4A and NS4B in the CP CSFV replicon is marked with an arrowhead at the left.

FIG. 6. Pulse-chase analyses of NS2 (A), NS3 (B), NS4A (C), NS4B (D), NS5A (E), and NS5B (F) after transfection of a CP CSFV replicon and after infection with non-CP CSFV. Metabolic pulse-labeling was performed with [³⁵S]methionine-cysteine at 8 h posttransfection of CP CSFV replicon RNA or at 12, 24, and 72 h postinfection with non-CP CSFV as indicated below each analysis. Pulse-chase analyses of NS3 (G) and NS5B (H) after infection with CP CSFV-JIV were performed 24 h postinfection with CP CSFV. After radiolabeling, SK-6 cells were harvested and used for immunoprecipitation (pulse) or incubated with DMEM enriched in unlabeled methionine/cysteine and harvested after the indicated time (chase). Specificity of applied MAbs is shown above each experiment. Labeled proteins were analyzed by SDS-PAGE and autoradiography. ¹⁴C-labeled marker proteins are indicated in kilodaltons on the left. The positions and identity of nonstructural proteins and respective precursor molecules are marked with arrowheads at the right. A high level of expression of NS3 is documented for the CP CSFV replicon, leading to efficient proteolytic maturation of NS5A and NS5B. Balanced generation of mature NS3 is observed throughout the monitored infection time and occurs primarily cotranslationally in non-CP CSFV-infected cells (B). Processing of the NS4-5 precursor molecule is slow, and concentrations of mature proteins are at the limit of detection in non-CP CSFV-infected cells (C, D, E, and F). In CP CSFV-JIV-infected cells, NS2-3 is rapidly processed and the mature NS3 (G) mediates a fast proteolytic processing of NS4-5 precursors to NS5B (H).

during the first chase period (0.5 h) indicated a further supply from the NS2-3 precursor. Thus, a posttranslational product-precursor relationship between NS3 and NS2-3 can be assumed. NS2-3 is slowly converted, with a half-life of about 5.5 h for non-CP CSFV (Fig. 7A). Our pulse-chase experiments at 12, 24, and 72 h after infection indicated that the concentration of mature NS3 stayed almost constant during infection, while

the synthesis of NS2-3 decreased before 72 h postinfection with non-CP CSFV (Fig. 6B).
The 170-kDa precursor consists of NS4A, NS4B, NS5A, and NS5B. In cells transfected with the CP CSFV replicon only the 170-kDa precursor was precipitated with NS4A- and NS4B-specific antibodies, but no products became apparent within a chase time of 24 h (Fig. 4D). CP CSFV repliconV₂₂₉₉M, har-

FIG. 6—Continued.

FIG. 7. Determination of the turnover kinetics of nonstructural protein precursors and mature nonstructural proteins of non-CP CFSV, a CP CFSV replicon, and CP CFSV-JIV. Radioactive signals of individual protein bands before chase (0 h) and after the indicated chase time (0.5, 1.5, 4.5, 12, and 24 h) were quantified by phosphorimaging. Intensity data for NS2-3 (A), NS4-5 (B), NS5A/B (C), NS3 (D), NS4A (E), NS5A (E), and NS5B (E) were calculated as relative signal intensity for non-CP CFSV, a CP CFSV replicon, and CP CFSV-JIV in percentages. The arithmetic average was calculated from multiple experiments for each time point. Dispersion of values within independent experiments is indicated by error bars. The relative intensity data were fit to a first-order decay function. The regression line is shown as a dotted line in each graph, and the respective formula is shown. The gradient of the regression line was used to calculate the protein's half-lives. The time of additional supply (around 1.5 h) was subtracted to calculate the half-life of mature proteins in the context of the CP CFSV replicon. Data from late chase time points (12 h and 24 h) was excluded from linear regression, because no linear correlation was found.

FIG. 7—Continued.

boring the single-amino-acid exchange $V_{2299}M$, allowed metabolic labeling of mature NS4A. The same mutation was tested before in the context of the genomic non-CP CSFV and did not cause apparent changes of the phenotype. The modified CP CSFV replicon $V_{2299}M$ was used only to analyze the biosynthesis of NS4A (shown in Fig. 6C). Very weak signals of mature NS4A were detectable in this experiment, which nevertheless allowed us to calculate a half-life of about 2 h for NS4A, taking additional generation from NS4-5 into account (Fig. 7E). The 170-kDa precursor was precipitated from non-CP CSFV-infected cells with NS4A- and NS4B-specific MAbs as well. Processing products, such as an uncleaved NS4A/B molecule or the mature proteins, were not observed (Fig. 6C and D). A

quick turnover of the NS4-5 precursor was documented with NS5-specific antibodies in cells transfected with CP CSFV replicon RNA. The calculated half-life of NS4-5 was 0.8 h (Fig. 7B), and the visible processing product NS5A/B was further processed, with a similar short half-life ($t_{1/2}$, 0.5 h), taking the additional supply from the precursor into account (Fig. 7C). NS5A-specific antibodies precipitated the 170-kDa NS4-5 precursor, the 130-kDa NS5A/B precursor, and mature NS5A with 56 kDa. Although large amounts of mature NS5A were detected, no molecular mass increase due to hyperphosphorylation was observed during chase time (Fig. 6E). Antibodies against NS5B gave similar results, revealing the 170-kDa NS4-5 precursor, the 130-kDa NS5A/B precursor, and a ma-

FIG. 8. Synopsis of CSFV nonstructural protein processing. Bars symbolize individual proteins. Designation, apparent molecular mass, and calculated half-life of the respective proteins are indicated above the bars.

ture NS5B of around 80 kDa (Fig. 6F) for the CP CSFV replicon. The predominant mature NS5A and NS5B displayed half-lives of 1.5 h and 2 h (Fig. 7E). Using the NS5-specific MAbs in pulse-chase experiments with non-CP CSFV-infected cells, a slower processing of the 170-kDa NS4-5 to 130-kDa NS5A/B was documented (Fig. 6E and F). A half-life of about 3 h was calculated for NS4-5 in non-CP CSFV-infected cells, and a significant increase of the NS5A/B molecule half-life ($t_{1/2}$, 2.5 h) was found as well (Fig. 7B and C). The turnover of both precursors was around three times faster in the CP CSFV replicon than in the non-CP CSFV (Fig. 8). In contrast to results with the replicon, the mature NS5B solely appeared in non-CP CSFV-infected cells and no signals of mature NS5A were revealed (Fig. 6E and F).

To show that processing and protein stability of CP pestivirus-infected cells are reproduced by the CP CSFV replicon system, we performed pulse-chase analyses of proteins from cells infected with the CP CSFV-JIV. Analysis with an MAb against NS3 (GL3p1) revealed that NS2-3 (125 kDa) is quickly processed to mature NS3 (75 kDa; Fig. 6G). A half-life of about 2.5 h was calculated for NS2-3 in CP CSFV-JIV-infected cells (Fig. 7A). Again, the signals of mature NS3 remained

strong during the chase period and a half-life of about 4 h was calculated (Fig. 6G). A pulse-chase analysis with the MAb against NS5B showed that the NS4-5 precursor with an apparent molecular mass of 170 kDa is quickly converted ($t_{1/2}$, ca. 1 h; Fig. 7B) to NS5A/B with an apparent molecular mass of 130 kDa (Fig. 6F). As seen in cells transfected with the CP CSFV replicon, the second precursor NS5A/B ($t_{1/2}$, ca. 0.8 h) is further processed to mature NS5B with a molecular mass of 80 kDa (Fig. 6H and 7C). The calculation of mature NS5B's half-life as 2.5 h (Fig. 7E) points out that not only processing but further protein stability of CP CSFV-JIV is reproduced by the CP CSFV replicon.

DISCUSSION

The life cycle of most positive-strand RNA viruses involves the proteolytic maturation of functional polypeptides by viral proteases after polyprotein translation (14). Viral proteases that mediate the generation of enzymatically active NSP represent a key element for the regulation of replication. Biosynthesis of pestivirus NSP is well studied only for CP BVDV (7), while the much more relevant non-CP field viruses of BVDV

and CSFV are poorly characterized. The cytopathogenic biotype of BVDV exhibits a deregulated replication (15), is unable to induce persistence, and therefore rapidly disappears in the population. We postulate that knowledge about the regulation of NSP biosynthesis in the non-CP biotype of pestiviruses is an important premise toward understanding the molecular basis of persistence. In this study, non-CP CSFV was chosen as a model for the non-CP biotype to investigate NSP biosynthesis in infected tissue culture cells, including a comparison to CP CSFV-JIV and a CP CSFV replicon. To ensure unambiguous detection, MAbs were developed against all NSP of CSFV that are involved in replication (NS2-NS5B).

We identified MAbs against NS2, NS4A, NS5A, and NS5B from our panels that specifically reacted with CSFV-infected cells. Using these MAbs along with an established MAb anti-NS3 the localization of NSP of non-CP CSFV and that of CP CSFV-JIV were compared in confocal laser scanning microscopy. The broad cytoplasmic distribution of NS2 and a focal perinuclear localization of NS5A were almost the same in non-CP CSFV- and CP CSFV-JIV-infected cells. Apparent differences were observed for the signals of NS3, NS4A, and NS5B (Fig. 3). In non-CP CSFV-infected cells a focal perinuclear shape was apparent for NS3, NS4A, and NS5B that in CP CSFV-JIV-infected cells was more broadly distributed. Whether these differences are real or just consequences of higher antigen concentrations in CP CSFV-JIV-infected cells cannot be determined at this point. The distribution is typical for the endoplasmic reticulum (ER), and thus an interaction of NS2-NS5B with these membranes is likely. In-depth colocalization studies with higher resolution and/or immune electron microscopy are required to determine the localization of each of the NSP in detail.

A complex pattern of high-molecular-weight precursors and mature NSP was identified in immunoblot analyses of SK-6 cells 48 h after infection with non-CP CSFV. NS2-3 was dominant, while minute amounts of NS2 and small quantities of NS3 appeared. The NSP downstream of NS3 were equally present as high-molecular-weight precursors (NS4A/B, NS4B-5A, NS5A/B, and NS4-5) and as mature forms (NS4A, NS5A, and NS5B). These immunoblot analyses present the total viral protein content in cells at 48 h postinfection with non-CP CSFV and include newly synthesized translational products as well as stable viral proteins accumulated over the entire infection time. The biosynthesis of viral NSP and precursor-product relationships was studied using metabolic radiolabeling techniques. Immunoprecipitation experiments with metabolically labeled NSP demonstrated an early generation of NS2-3, NS3, and the precursor molecules NS4-5, NS5A/B, and NS4B-5A. Tracing these precursors at different time points postinfection with non-CP CSFV, we observed a continuous slow conversion from NS4-5 up to mature NS5B. Mature NS4A, NS4B, and NS5A were not detected in the pulse-chase experiments with non-CP CSFV-infected cells. The half-lives calculated for NS2-3 ($t_{1/2}$, 5.5 h), NS4-5 ($t_{1/2}$, 3 h), and NS5A/B ($t_{1/2}$, 2.5 h) were similar at different time points after infection, indicating a constantly slow processing throughout the infection with non-CP CSFV.

To compare the observed slow processing of the nonstructural proteins of non-CP CSFV with a homologous cytopathogenic counterpart, a CP CSFV replicon was constructed. Anal-

ysis of NS4A, NS4B, NS5A, and NS5B synthesis in cells transfected with this CP CSFV replicon revealed a significant acceleration of NSP processing over that with non-CP CSFV. Immunoprecipitations visualized the synthesis of equal quantities of precursor molecules (NS4-5, NS5A/B, and NS4B-5A) and mature NS5A and NS5B in the CP CSFV replicon during the pulse time. The substantially shorter half-lives of the precursors NS4-5 ($t_{1/2}$, 0.8 h) and NS5A/B ($t_{1/2}$, 0.5 h) in the CP CSFV replicon system indicate a positive correlation between NS3 synthesis and maturation of all downstream NSP that are involved in replication. This finding is supported by analyses of NSP processing in cells infected with CP CSFV-JIV (10). Immunoprecipitation experiments demonstrated equally short processing times of the NS4-5 region for CP CSFV-JIV and the CP CSFV replicon. Again, the generation of mature NS5A and NS5B was visible within the pulse-labeling time of 1 h. Pulse-chase experiments showed that the NS2-3 molecule was converted with a half-life of about 2.5 h in CP CSFV-JIV, while substantial amounts of mature NS3 had already been generated cotranslationally. Kinetic analyses with an MAb against NS5B demonstrated short apparent half-lives of NS4-5 ($t_{1/2}$, 1 h) and NS5A/B ($t_{1/2}$, 0.8 h) in CP CSFV-JIV-infected cells, as seen in the CP CSFV replicon. This can be taken as evidence that, except for the efficient *cis* cleavage between NS2-3 and NS4A, the serine protease of the NS2-3 is a less active proenzyme that requires activation by NS2/JIV-mediated maturation for its *trans*-cleavage activity. Elegant experiments by Tautz et al. have shown that a glutathione-S-transferase-NS3 fusion is enzymatically active and hence a free N terminus of NS3 is not required for its protease activity (29). However, specific inhibitory interactions between domains of NS2 and the NS3 protease, steric interference, or the membrane association of NS2-3 may contribute to an impairment of NS3 protease *trans*-cleavage activity.

The significantly higher expression of mature NSP together with the shorter half-life of precursors facilitated the calculation of mature NSP's half-lives in the CP CSFV replicon and in CP CSFV-JIV-infected cells. Therefore, the time of additional supply (around 1.5 h) was subtracted from the apparent half-lives of these molecules. The detection of mature NS3 ($t_{1/2}$, 4 h), NS4A ($t_{1/2}$, 2 h), NS5A ($t_{1/2}$, 1.5 h), and NS5B ($t_{1/2}$, 2 h) in immunoblot analyses of non-CP CSFV-infected cells is therefore very likely a consequence of accumulation of these stable processing products.

The genomic organization of the NSP regions of HCV and CSFV (and other pestiviruses) is alike with regard to membrane topology, processing events, and enzymatic functions. The study of HCV-related pestiviruses may provide insights into principles of regulation of *Flaviviridae* that cause persistent infections. NS2 to NS5B are released by the virus-encoded NS2 autoprotease together with the major NS3 protease in both genera, hepacivirus and pestivirus. Striking differences have been found in the processing products between CP BVDV and HCV. The NS2-3 cleavage is very efficient in HCV, and a NS2-3 precursor is not detectable (25). A complex processing of NS3 to NS5B was observed using a recombinant vaccinia expression system for HCV NSP. The processing at NS3-NS4A and NS5A-NS5B sites occurs rapidly. In contrast, a delayed processing at NS4A-NS4B and NS4B-NS5A sites generates relatively stable NS4A/B-NS5A processing intermediates

(2). A kinetic model of NSP processing and the stability of mature NSP of HCV was established with the help of self-replicating HCV RNAs that induced no cytopathogenic effect in their host cells. Analyses of these subgenomic HCV replicon-bearing cells confirmed the mode of processing found with the vaccinia expression system. This replicon system enabled the calculation of mature HCV NSP. Calculated half-lives of HCV NSP ranged from 10 to 16 h, showing a marked stability of these proteins (20). The study of NSP processing in novel cell culture systems for the replication of genomic HCV RNAs that produce infectious HCV particles (33) has yet to be done.

Results from kinetic analyses of NSP processing in non-CP CSFV revealed that NS2-3 cleavage is delayed, while the *cis* NS3-NS4A cleavage occurs cotranslationally. A precursor-product relationship for NS4-5 and NS5A/B was apparent, and both molecules were relatively stable. The appearance of an NS4B-NS5A precursor suggests that alternative cleavages, such as initial processing at the NS5A-NS5B site, exist. The processing of the NS4A/B precursor molecule appears to be rather efficient, because it could not be detected in the pulse-chase analyses. A synopsis of precursor-product relationship of the CSFV NSP region, including observed molecular masses and calculated half-lives, is depicted in Fig. 8.

Using newly developed MAbs, we were able to document a delayed processing for the entire nonstructural protein region of a non-CP pestivirus compared to those of a homologous CP pestivirus and a homologous CP replicon, except for the NS3-NS4A *cis* cleavage. Previous data reported a rapid processing of NS2-3 and NS4-5 for the CP biotype of BVDV (7) and a JIV-dependent restriction of the NS2-3 cleavage for non-CP BVDV (15). The JIV-mediated enhancement of NS2-3 cleavage in CSFV has been shown by the genetically engineered CP CSFV-JIV (10). According to previous data on non-CP BVDV, the concentration of JIV alone is sufficient to moderate the release of NS3 that occurs efficiently only at very early stages of infection in non-CP BVDV-infected cells (15). This is probably not the case in non-CP CSFV-infected cells, where a constant generation of NS3 is observed over 72 h after infection. Nevertheless the results for non-CP CSFV strongly confirm the model that the autoproteolytic NS2- and JIV-dependent cleavage of NS2-3 is an activation step for the serine protease moiety of NS3. As NS3 is quite stable ($t_{1/2}$, 4 h), a constant supply of newly generated NS3 occurs at about the same low speed. Future experiments will address the slow processing of NS2-3 in non-CP CSFV and determine the influence of JIV on the intracellular levels of NS3 in non-CP CSFV-infected cells.

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