Intracellular processing of the Sendai virus C′ protein leads to the generation of a Y protein module: Structure–functional implications

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Abstract The Sendai virus "*C-proteins*" (C′, C, Y1 and Y2) are a nested set of non-structural proteins. The shorter Y proteins arise in vivo both by de novo translation initiation and by proteolytic processing of C′. In this paper, we demonstrate that C′ but not C (differing only by 11 N-terminal amino acid) serves as an efficient substrate for intracellular processing. However, processing can be mimicked in vitro by the addition of endopeptidases. Under conditions of limited proteolysis we observed that in a fraction of the C′ protein the Y region exists as a proteinase resistant core. This core was conserved in the C protein. We propose that C′ functions as a Pro-protein delivering the Y module to a specific intracellular location.

Keywords: Protein processing; Translation initiation; Viral non-structural proteins

1. Introduction

The Sendai virus "*C-proteins*" (C′, C, Y1 and Y2) represent a group of viral non-structural proteins that although not essential for growth in cell culture, play key roles in: (a) modulating the read-out from the viral genome [1], (b) maintenance of genomic stability [2], (c) viral particle budding [3], and, (d) modulating the host cell response during infection [4,5]. They are expressed from the P/C mRNA that codes for no less than six different polypeptide chains (namely, C′, P, C, Y1, Y2 and X) [6,7]. The initiation sites were mapped by in vitro mutagenesis [8]. The largest of the four "*C-proteins*" starts at an ACG codon at position 81 (ACG^{81}) [9,10], with the others initiating at AUG^{114} (C), AUG^{183} (Y1) and AUG^{201} (Y2) (Fig. 1A). Changing ACG^{81} to AUG (AUG81) ablated all translation from the second and third sites [9]. Nonetheless, Y expression continued, suggesting that ribosomes had scanned non-linearly to these start codons. However, a number of in vivo observations were difficult to reconcile with a simple initiation model. Firstly, Y expression was AUG codon independent (an event not observed in vitro) [8,11,12]. Secondly, deletion mutants were identified that significantly altered Y expression in a manner that was independent of the Y start codons (but not in vitro). In the AUG81 background, deletion of nucleotides 189–197 (Δ9) increased Y by up to 10-fold. Two mutations severely perturbed Y expression. The first deleted 24 nts downstream of the Y2 start codon (Δ10), whilst the second added 6 amino acid (aa) to the extreme N-terminal of C′ [11,13].

The Y proteins can arise both via de novo translation initiation from AUG^{183}/AUG^{201} and also via processing of C′ [13]. This processing occurs in living cells but not in vitro, reconciling the conflicting in vivo versus in vitro observations. In this report, we demonstrate that processing can be mimicked in vitro using endoproteinases suggesting that Y represents a proteinase resistant core. Curiously, this core also exists in the shorter C protein even though it does not serve as an efficient processing substrate in cells. We propose that this failure to process C is linked to its different intracellular location.

2. Materials and methods

2.1. DNA constructions

Constructs AUG81, $C'^{\Delta 9}$, $C'^{\Delta 9YCys}$ (Y start codons have been changed to UGU), $C'^{\Delta 10}$, $C'^{\Delta 9+6}$ (6 aa have been fused to the N terminal of C′) are described in [11,13]. The regions from $AUG^{81/C'}$ to $AUG^{183/Y1}$, $AUG^{114/C}$ to $AUG^{183/Y1}$ and $AUG^{81/C'}$ to $AUG^{114/C}$ were amplified by PCR and substituted into the pEBS-PL AUG81-GFP (C′-GFP) clone [13] as a *Nco*I/*Xba*I fragment to generate pEBS PL (1–35)-GFP, (12–35)-GFP, and (1–12)-GFP, respectively (numbers refer to the aa positions in C′).

2.2. Cell culture, transient transfection and metabolic labelling

Cell culture, transfections, metabolic labelling and pulse chase experiments were performed as previously described in [13].

2.3. In vitro transcription and translation

Run-off capped transcripts were transcribed with the bacteriophage T7 RNA polymerase in 800 μM ATP/CTP/UTP, 400 μM GTP and 2 mM m^7GpppG cap analogue (Ambion). Capped mRNAs (50 μg/ml) were translated in a rabbit reticulocyte lysates (RRL) (Promega) in the presence of 0.5 mM MgOAc, 75 mM KCl, 20 μM aa (minus methionine) and 0.5 mCi/ml of ^{35}S-translabel. The mixture was incubated for 1 h at 30 °C. Translation products were either analysed directly or immunoprecipitated with an anti-HA monoclonal antibody (Ab 16b12; Eurogentec).

2.4. Limited proteolysis

RNA transcripts from clones coding for AUG81, $C'^{\Delta 9YCys}$, $C'^{\Delta 10}$, C^{wt} and $C'^{\Delta 9+6}$ were translated in a RRL, or transiently expressed in HeLa cells. Extracts were treated with chymotrypsin at the doses indicated in the figures. The proteinase was stopped by the addition of 1% SDS and heating at 65 °C for 5 min. Samples were diluted 10-fold in lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 5 mM EDTA, and 0.5% NP40) before immunoprecipitating [13].

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Abbreviations: RRL, rabbit reticulocyte lysates; STAT, signal transducers and activators of transcription; aa, amino acid

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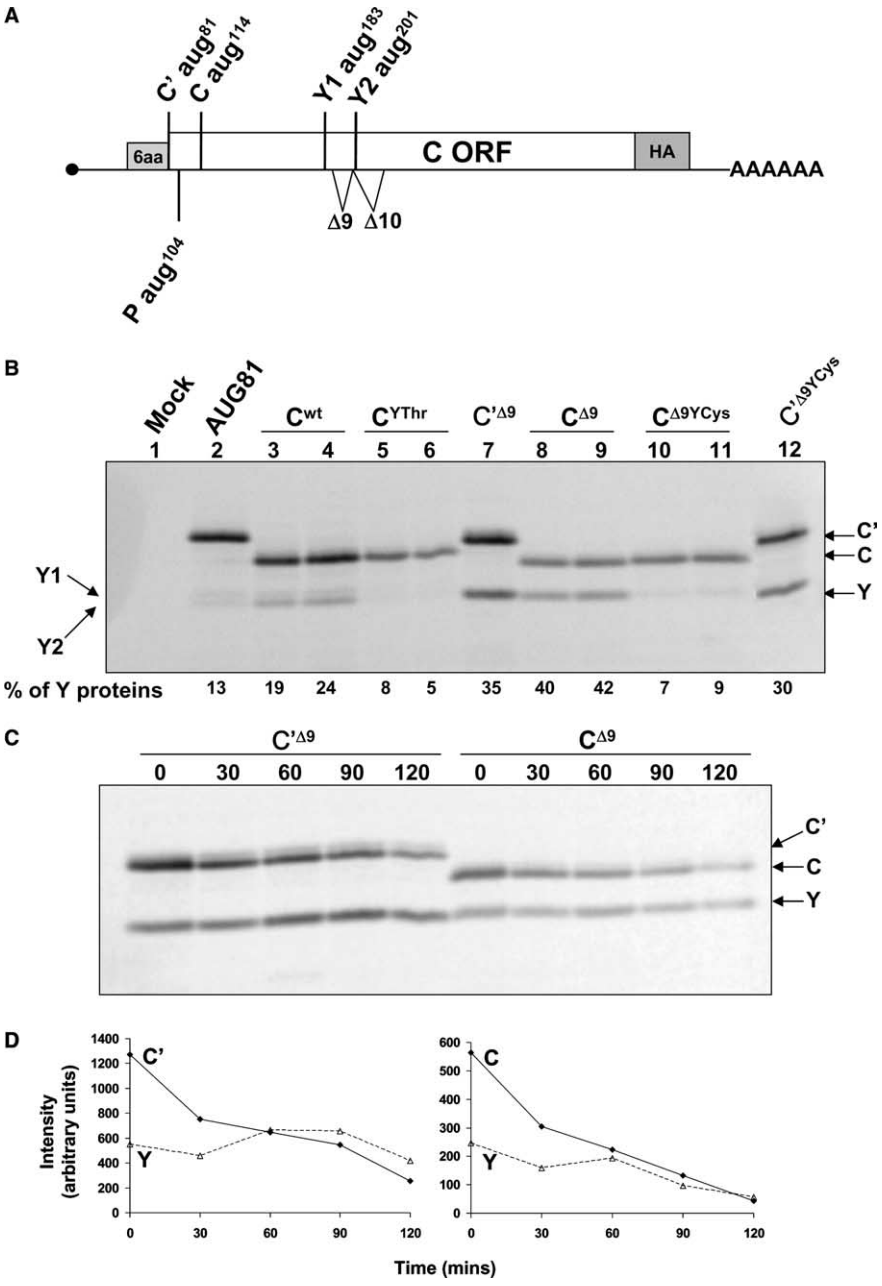

Fig. 1. C protein is a poor proteolytic substrate in vivo. (A). Schematic representation of the C ORF in the background AUG81 indicating the initiation sites. The AUG codon for P is indicated below the line because it is in a different ORF. HA refers to the triple HA epitope tag. The position of the 6 aa N-terminal extension and the sites of the Δ9 and Δ10 deletions are also indicated. (B) HeLa cells infected with vaccinia-T7 were transfected with the clones indicated above the panel. In the mock control, cells were transfected with an empty vector. Cells were labelled with ^{35}S translabel at 18–20 h postinfection. Cytoplasmic extracts were immunoprecipitated with an anti-HA antibody. Immunoselected proteins were resolved on a 15% SDS–polyacrylamide gel and visualised by fluorography. Bands were quantitated on a phosphorimager and the amount of Y is indicated below the panel as a percentage of the total protein immunoprecipitated. (C) Transfected HeLa cells expressing $C'^{\Delta 9}$ or $C^{\Delta 9}$ were metabolically labelled with ^{35}S translabel for 1 h at 18 h postinfection and then chased for the times (min) indicated above the panels. Proteins were immunoselected and analysed. The results are depicted graphically below the gels.

2.5. Antibodies and indirect immunofluorescence assay

HeLa cells were fixed and permeabilised in 3% formaldehyde/PBS during 20 min and 0.05% saponin/PBS during 5 min. They were then washed three times in PBS. The anti-HA mouse antibody was used at a 1:100 dilution in PBS. Coverslips were incubated with the antibody for 20 min at room temperature. After three PBS washes, the secondary antibody conjugated with alexa-568 (mouse) was added at a dilution of 1:200, for 20 min at room temperature. Confocal microscopic analysis was performed on a confocal laser scan fluorescence-inverted microscope (LSM 410, Zeiss).

3. Results

3.1. Y Proteins in vivo do not arise via processing of the C protein

We tested whether the shorter C protein served as an effective intracellular processing substrate by applying the criteria previously employed [13], namely: (a) does the Δ9 deletion produce an increase in Y expression, and (b) is Y expression inde-