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Molecular Dissection of the Vaccinia Virus I7L Core Protein Proteinase

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The vaccinia virus I7L gene product is predicted to be a cysteine proteinase and is demonstrated in this study to be responsible for cleavage of each of the three major core protein precursors (P4a, P4b, and P25K) in vivo. Mutagenesis of the putative catalytic triad of I7L or of the cleavage sites in the core protein precursors inhibits processing. A truncated protein lost the ability to cleave the core protein precursors.

Vaccinia virus (VV) is a large double-stranded-DNA virus with a cytoplasmic site of replication. It has over 200 open reading frames (ORFs) and has been extensively used as a eukaryotic cloning and expression vector and for vaccine research. VV is closely related to variola virus, the causative agent of smallpox, and therefore is of interest as a surrogate target in the development of antiviral drugs and vaccines. It is therefore of interest that the gene product of the I7L ORF of VV, which is predicted to be the core protein protease (2), shares 99% identity with the homologous K7L gene of variola major virus.

Most viruses, including poliovirus, human immunodeficiency virus, and adenovirus, use posttranslational proteolytic processing as an essential step in their replication cycles (8). Therefore, it was not surprising to discover that proteolytic maturation of orthopoxvirus core proteins appears to be required for infectious progeny to be produced (6). Three of the major structural proteins found within the mature VV virion core, 4a, 4b, and 25K, were known to be produced from higher-molecular-weight precursors at late times during infection (12). VanSlyke et al. (13, 14) demonstrated that a large number of VV core proteins, including 4a, 4b, and 25K, appear to be processed via a common morphogenic cleavage pathway that is intimately linked with virion assembly and maturation. Cleavage of the precursors occurs only within the context of the maturing virion. All of the precursor proteins appear to be cleaved at a novel Ala-Gly-Xaa motif. This motif is distinct from that utilized in any other viral system, although some of the cysteine proteinases identified in other systems cleave polyproteins at Gly-Gly-Xaa sites, as demonstrated by the yeast cysteine protease (9, 10), the adenovirus protease (15, 3, 5), and the African swine fever virus (ASFV) protease (1).

The gene product of the I7L ORF of VV was originally identified as a putative proteinase due to its homology to an ubiquitin-like proteinase in yeast (9) and was recently shown to be one of the proteinases responsible for cleavage of the VV core proteins (2). While there is a relatively detailed understanding of the *cis*-signals (sequences and protein structure characteristics) that direct the cleavage of the core protein precursors, relatively little is known about the enzyme that carries out these reactions. It is not known whether the entire I7L protein is required for recognition and cleavage of the core precursor proteins or if just the predicted catalytic domain is required. Is I7L capable of cleaving each of the core protein precursors, and does cleavage occur preferentially at Ala-Gly-Ala versus Ala-Gly-Ser and Ala-Gly-Thr sites? Is there a catalytic triad and are other conserved residues essential for activity? The results obtained show that intact I7L is necessary and sufficient to direct cleavage of each of the three major core protein precursors and that mutagenesis of either the putative catalytic triad of I7L or of the Ala-Gly-Xaa sites in the precursor proteins abolishes this activity.

The VV 17L ORF is predicted to encode a 423-amino acid protein with the catalytic domain located toward the carboxy terminus of the protein. Figure 1 shows a predicted hydrophobicity plot of the I7L protein made by using the Kyte-Doolittle program. The residues above the zero line are hydrophobic, and those beneath are hydrophilic. There are several hydrophobic domains near the amino terminus and the carboxy terminus of the protein. The positions of the residues of the putative catalytic triad (H, D, and C) are indicated, as are the positions of four other highly conserved amino acids (W, D, Q, and G). Also shown is the position of the *ts*16 mutation, where a proline was altered to a leucine (7), creating a temperature-sensitive virus capable of growth at 31 but not at 41°C. The *ts*16 virus was originally isolated by Condit et al. (4). Shown below the hydrophobicity profile are representations of the variola virus, camelpox, and monkeypox enzymes with positions of variance from VV I7L indicated with bars, which show that these enzymes are virtually identical to VV I7L and that the residues of the putative catalytic triad are conserved. The region within the I7L ORF with homology to the ASFV core proteinase is near the C terminus and overlaps the location of the putative catalytic triad. To determine if the N-terminal portion of the protein is required for activity, a truncated I7L was created, cutting off the N-terminal region up to amino acid residue 228. This process was done to remove both the N-terminal hydrophobic region and the region of the protein that was previously determined to have similarity to a topoisomerase. Li and Hochstrasser (9) and Andres et al. (1) have iden-

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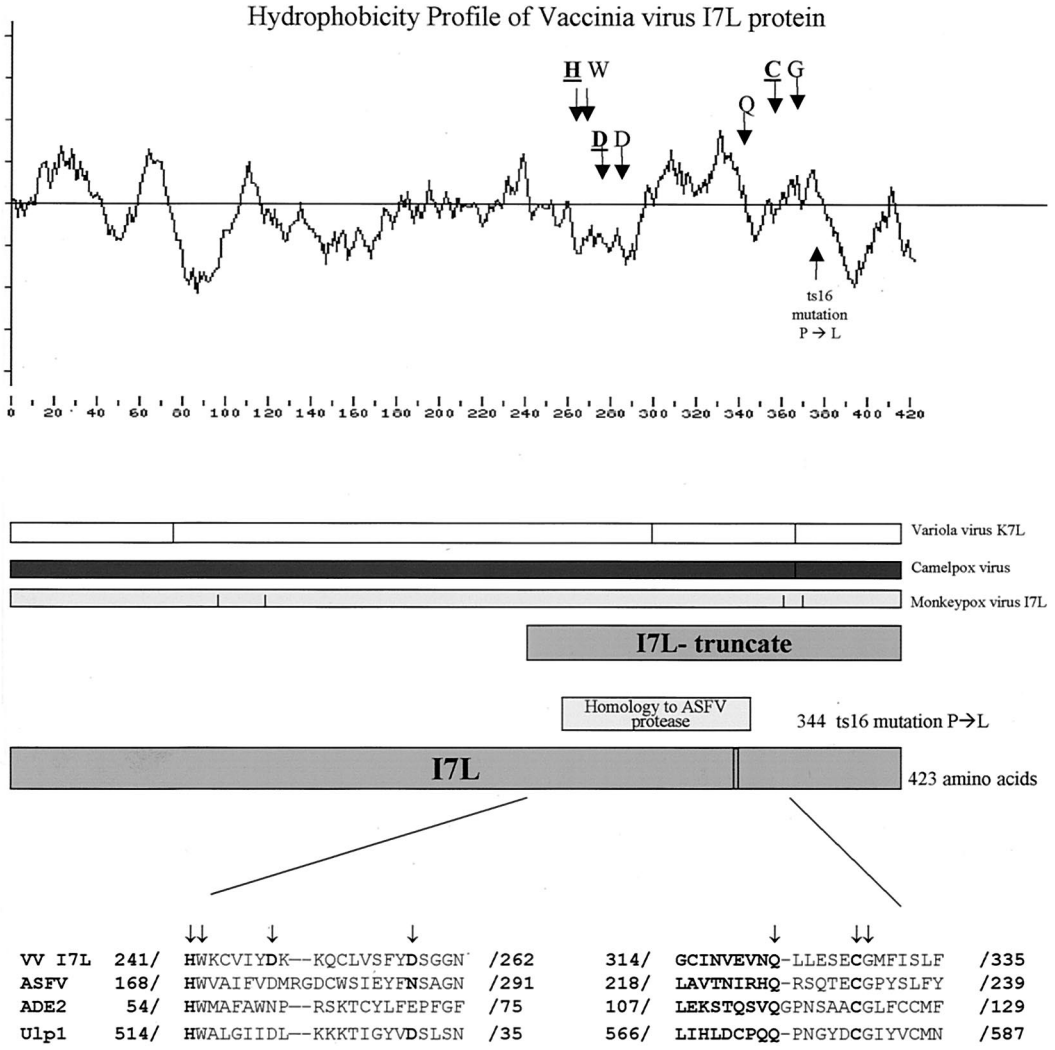

FIG. 1. Characterization of I7L and predicted hydrophobicity plot of the I7L protein made with the Kyte-Doolittle program. The positions of seven highly conserved amino acids, including the putative catalytic triad, and the position of the *ts*16 mutation are indicated by arrows. The positions of the amino acids are indicated on the *x* axis. Sequence similarity to the corresponding gene in variola virus, camelpox virus, and monkeypox virus is indicated by rectangles underneath the plot, with differing amino acids indicated at the correct positions by black bars. Sequence similarity of the conserved catalytic domain between VV I7L, the ASFV protease, adenovirus protease (ADE2), and a yeast cysteine protease (Ulp1) is indicated at the bottom of the figure, with arrows pointing at the highly conserved amino acids.

tified a conserved catalytic core domain between VV I7L, the ASFV protease, the adenovirus protease, and the *Saccharomyces cerevisiae* protease with several highly conserved amino acids. This domain is indicated in Fig. 1.

In previous work, we have shown that I7L is capable of cleaving P25K at the AGA and AGS sites. However, it was not known whether this cleavage reaction was specific to the P25K substrate or whether I7L was capable of cleaving the other core protein precursors. To determine whether I7L is responsible for cleavage of each of the three major core protein precursors, an in vivo *trans*-processing assay was utilized, where cells were infected with *ts*16 at the nonpermissive temperature and cotransfected with plasmid-borne substrate and enzyme. Both the substrate proteins and I7L protease were constitutively expressed in vivo by using a synthetic early-late promoter. Each core protein precursor-expressing plasmid was designed to express a Flag epitope on the C terminus for detection by Western blotting and differentiation from the analogous gene product expressed from the viral genome. Figure 2 is a map of the three major core protein precursors, P4a, P4b, and P25K, which are products of the A10L, A3L, and L4R ORFs, respectively, with the previously determined cleavage sites indicated on them. These cleavage sites have all been mapped to an Ala-Gly-Xaa motif (14, 16). P4a is the largest precursor protein, with a molecular size of 98 kDa, and contains both an Ala-Gly-Ser and an Ala-Gly-Thr cleavage site in the C-terminal region of the protein. P4b is a 71-kDa polyprotein with an N-terminal Ala-Gly-Ala site, and P25K is a 28-kDa polyprotein with both Ala-Gly-Ser and Ala-Gly-Ala cleavage sites in the N-terminal region of the protein. Also indicated are the relative sizes of the plasmid-borne I7L protein (pI7L) and the truncated I7L protein, which was truncated at amino acid 228,

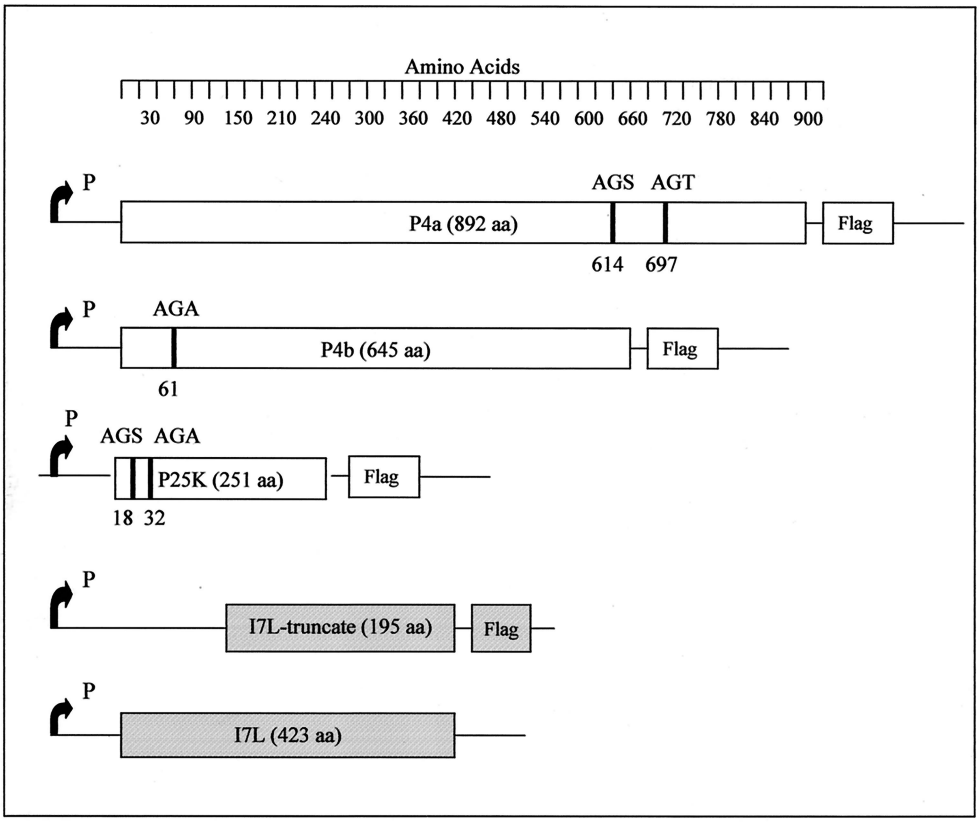

FIG. 2. VV core protein cleavage sites. Schematic representation of the three major core protein precursors (P4a, P4b, and P25K) along with full-length and truncated I7L. The positions of the AGX cleavage sites are indicated by the amino acid number of the glycine.

leaving residues 229 through 423, with a reengineered start site.

Cleavage of P25K by I7L has been shown previously (2), but here we demonstrate that I7L is capable of directing cleavage of the other core protein precursors as well. BSC_{40} cells (11) were infected with VV *ts*16 at a multiplicity of infection of five and transfected with 10 μg of plasmid DNA containing either I7L, P4b, P4a, or a mixture of these via a liposome-mediated transfection protocol. The cells were harvested at 24 h postinfection, and the extracts were analyzed by Western blotting with anti-Flag antisera. Figure 3A indicates that I7L cleaves P4b from its precursor form to the mature processed form (Fig. 3A, lane 2) but that when the histidine residue 241 (a member of the putative catalytic triad) of I7L is mutated to an alanine, this cleavage is no longer observed (Fig. 3A, lane 3). Lanes 1 and 4 are controls showing P4b and P4bIDI expressed alone. When the AGA site of P4b is mutated to IDI residues, no cleavage by I7L is observed (Fig. 3A, lane 5). Lane 6 is a

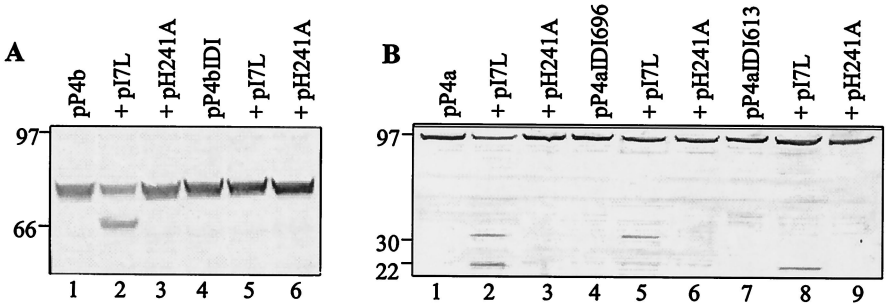

FIG. 3. Proteolytic processing of the core protein precursors. BSC_{40} cells were infected with VV *ts*16 and transfected with plasmids containing either I7L, P4a, P4b, or a mixture of these. Cells were harvested 24 h postinfection, and the extracts were analyzed by Western blot with anti-Flag antisera. (A) Processing of P4b. In each lane, cells are infected with *ts*16 and then transfected with either substrate alone or with substrate plus enzyme. The substrate is pP4b or pP4bIDI (where the AGA site is mutated to an IDI), and the enzyme is either pI7L or pH241A (pI7L with His 241 mutated to Ala). (B) Processing of P4a. In each lane, cells are infected with *ts*16 and then transfected with either substrate alone or with substrate plus enzyme. In this case, the substrate is either pP4a, pP4aIDI696 (P4a with the AGT site mutated to an IDI), or pP4aIDI613 (P4a with the AGS site mutated to an IDI).

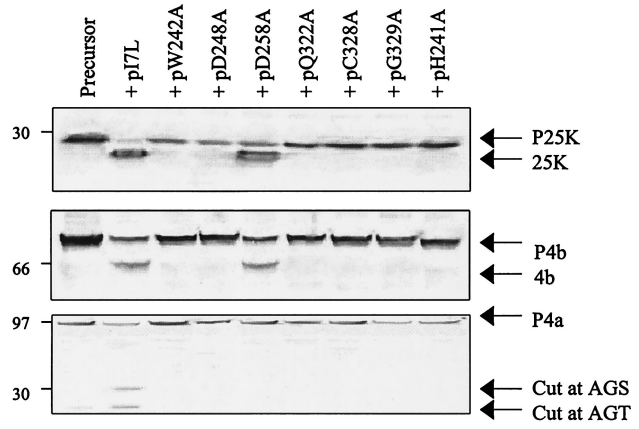

FIG. 4. Ability of mutant I7L enzymes to cleave the core protein precursors. BSC_{40} cells were infected with VV *ts*16 and transfected with plasmids containing either P4a, P4b, or P25K and cotransfected with either pI7L or one of the seven mutant I7L plasmids. Cells were harvested 24 h postinfection, and the extracts were analyzed by Western blot with anti-Flag antisera to determine cleavage of the precursor protein. The top and middle panels show P25K and P4b, respectively, transfected with each mutant I7L plasmid, and the bottom panel shows P4a transfected with each mutant enzyme.

TABLE 1. Rescue of the growth and proteolytic processing activity of vaccinia virus *ts*16 by I7L and I7L mutants[a]

Plasmid	Amt of VV *ts*16 (titer)	Amt of folding rescued
*ts*16	1.2×10^6	1
pI7L	9.2×10^6	7.7
pI7L-T[b]	1.4×10^6	1.2
pH241A	1.2×10^6	1.0
pW242A	1.3×10^6	1.1
pD248A	2.4×10^6	2.0
pD258A	4.3×10^6	3.6
pQ322A	1.3×10^6	1.1
pC328A	1.2×10^6	1.0
pG329A	1.3×10^6	1.1

[a] BSC_{40} cells were infected with VV *ts*16 and transfected with the indicated plasmid at the nonpermissive temperature. Cells were harvested 24 h postinfection, and the titer of the virus was determined in 6-well plates to determine the ability of the indicated plasmid to rescue viral replication. The amount of folding rescued was determined by dividing the titer of the mutant plasmid by the titer of *ts*16.

[b] pI7L-T, truncated pI7L.

final control showing that with mutant P4b and mutant I7L no cleavage products are observed, indicating that other proteases in the virus or cells are not causing the cleavage reactions. This experiment was repeated with the P4a polyprotein, as shown in Fig. 3B. When P4a is expressed alone (Fig. 3B, lane 1), it runs at its mature size of 98 kDa, but when I7L is transfected with P4a, two cleavage products are observed at around 22 kDa and 32 kDa (Fig. 3B, lane 2), indicating that cleavage is occurring at both the AGS and AGT sites. Mutation of I7L abolishes this cleavage (Fig. 3B, lane 3). When the AGS site of P4a is mutated to an IDI and the transfection is carried out with I7L, only one band at around 22 kDa is observed exhibiting cleavage at only the AGT site (Fig. 3B, lane 8). A similar result is obtained when the AGT site is mutated to an IDI and transfected with I7L (Fig. 3B, lane 5), where a band at around 30 kDa is observed, showing that cleavage is blocked at the IDI site but is still occurring at the AGS site. This finding shows that the catalytic activity of I7L, as well as the presence of the authentic cleavage sites, is necessary for cleavage to occur.

To further characterize whether the catalytic domain of the I7L protein was necessary and sufficient for recognition and cleavage of the core protein precursors, a truncated I7L was created with the N terminus removed up to amino acid 228. This truncated I7L was cloned into a plasmid behind the synthetic early-late promoter. The *trans*-processing assay was repeated with this I7L truncate. The I7L truncate was unable to cleave either P4a, P4b, or P25K, indicating that this region is essential for activity (data not shown).

To determine which of the seven previously indicated conserved amino acids is necessary for catalytic activity of I7L, site-directed mutagenesis was performed on each in turn to mutate the residue of interest to an alanine. Transient-expression assays were performed to test the activity of the mutant proteins on each of the core protein precursors. Briefly, cells were infected with VV *ts*16 at a multiplicity of infection of five and transfected with 10 μg of plasmid DNA by using DMRIE-C liposome-mediated reagent (Invitrogen). Virus-infected cells were harvested 24 h postinfection and centrifuged, and the resuspended pellet was subjected to three cycles of freeze-thaw to release the virus from the cell. The supernatant was used for analysis by polyacrylamide gel electrophoresis. Western blotting was performed with anti-I7L serum to test for expression of the enzyme and with Flag monoclonal antisera to check for processing of the precursor proteins. Each of the mutant I7L enzymes was expressed equally well (data not shown). Figure 4 shows each core protein precursor transiently expressed along with I7L and each mutant I7L. The top panel shows results with P25K, the middle panel shows results with P4b, and the bottom panel shows results with P4a. As shown on Fig. 4, full-length I7L is capable of cleaving each precursor protein, but when H241, W242, D248, Q322, C328, or G329 is mutated to an alanine, this cleavage is lost. The only mutant I7L that was still capable of cleavage was D258 mutated to an alanine, signifying that this mutant might not be a member of the catalytic triad. Cotransfection with pD258A showed that this protein was still capable of cleaving P25K and P4b, although cleavage of P4a was not seen.

To test whether I7L is capable of rescuing the growth and proteolytic processing activity of the *ts*16 virus, the virus was either grown alone, in the presence of transfected full-length I7L, in the presence of transfected mutant I7L, or with truncated I7L at the nonpermissive temperature. After 24 h of infection, the virus-infected cells were harvested, and then the titers of the virus were determined to determine rescue. As shown in Table 1, full-length I7L was capable of rescuing the growth of *ts*16, indicating that I7L is indeed the gene product that is mutated in *ts*16. Neither the truncated I7L nor any of the mutant I7L enzymes was capable of rescuing growth of *ts*16 except for pD248A and pD258A.

The identity of the protein responsible for cleavage of the VV core protein precursors has recently been identified as the gene product of the I7L ORF (2). In this report, we further characterized the properties of this protein. The data reported here utilizing an in vivo *trans*-processing assay with an epitope-

tagged substrate and plasmid-borne enzyme indicate that I7L is capable of driving the cleavage reaction and further verify that it is the viral core protein proteinase. Mutational analysis has shown that for this reaction to occur, catalytic activity of I7L is required and the authentic cleavage site has to be present in the substrate. This appears to be a global effect in that I7L is able to cut at the authentic Ala-Gly-Ala sites of P4b and P25K as well as the Ala-Gly-Ser and Ala-Gly-Thr sites within P4a, although it appears that the cleavage of P4a and P4b is less efficient than that of P25K. Whether this finding reflects natural cleavage kinetics or fast versus slow cleavage sites or whether it is a consequence of the transient expression system remains to be determined.

In this study, we utilized an in vivo assay to look at the proteolytic processing of core protein precursors. This assay does not enable the identification of potential cofactors or the biochemical parameters of the cleavage reaction. While an in vitro transcription-translation system would be helpful to show if other viral proteins or induced cellular proteins are required for this processing, we have not yet succeeded in establishing this assay, which may be due to our incomplete understanding of the reaction or the hydrophobic nature of the I7L gene product.

The I7L protein is characterized as a cysteine protease because mutation of the histidine, cysteine, and aspartic acid residues eliminates proteolytic activity. In addition, the other highly conserved residues in the catalytic core domain (W242, Q322, C328, and G329) are all necessary for proteolysis to occur. Of the conserved amino acids mutated, the only residue that was not found to be essential for proteolysis was D258. Truncation of the protein at amino acid 228 results in loss of processing of the core proteins, indicating that the amino terminal portion of the protein is necessary for either recognition or catalytic activity. It is not clear if the truncated protein is inactive because of the loss of essential activities inherent in this region or if this inactivity is due to an indirect effect on protein structure that disrupts essential folding needed by the catalytic domain. A series of site-specific mutants and truncations will be required to address this issue. One attractive and testable hypothesis might be that the N-terminal region of the protein has DNA-binding activity necessary to ensure virion packaging of the proteinase activity.

Regardless of the type of proteolytic maturation utilized by the virus during maturation, it is essential that the activity of the viral proteinases be regulated to ensure efficient production of infectious progeny virions. It will be of interest to discover the trigger that signals the activation of the VV I7L protease and how it is regulated to carry out its activity at a distinct point in the virus life cycle.

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