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The Murine Cytomegalovirus pp89 Immunodominant H-2L<sup>d</sup> Epitope Is Generated and Translocated into the Endoplasmic Reticulum as an 11-Mer Precursor Peptide<sup>1</sup>

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The 20S proteasome is involved in the processing of MHC class I-presented Ags. A number of epitopes is known to be generated as precursor peptides requiring trimming either before or after translocation into the endoplasmic reticulum (ER). In this study, we have followed the proteasomal processing and TAP-dependent ER translocation of the immunodominant epitope of the murine CMV immediate early protein pp89. For the first time, we experimentally linked peptide generation by the proteasome system and TAP-dependent ER translocation. Our experiments show that the proteasome generates both an N-terminally extended 11-mer precursor peptide as well as the correct H2-L<sup>d</sup> 9-mer epitope, a process that is accelerated in the presence of PA28. Our direct peptide translocation assays, however, demonstrate that only the 11-mer precursor peptide is transported into the ER by TAPs, whereas the epitope itself is not translocated. In consequence, our combined proteasome/TAP assays show that the 11-mer precursor is the immunorelevant peptide product that requires N-terminal trimming in the ER for MHC class I binding. *The Journal of Immunology, 2001, 167: 1515–1521.

The importance of the proteasome system in MHC class I Ag presentation is well established (reviewed in Ref. 1). The 20S proteasome, the proteolytic core of this protein degradation system, is an abundant cellular multisubunit proteasome complex that structurally resembles a cylinder of four stacked heptameric rings composed of two different sets of protein subunits (a<sub>1</sub>-7, b<sub>1</sub>-7) (2, 3). The catalytic sites are located within the two inner rings of the 20S proteasome complex and are formed by three constitutive β subunits, β<sub>1</sub>, β<sub>2</sub>, and β<sub>5</sub> (i.e., delta, z, MB1). Importantly, the active site bearing β subunits can be replaced by IFN-γ-inducible homologous subunits (β<sub>2</sub>1/LMP2, β<sub>2</sub>beta/MECL1, β<sub>5</sub>/LMP7), resulting in the formation of the, so-called, immunoproteasome. Another IFN-γ-inducible component of the proteasome system is the proteasome modulator complex PA28 (4–6), composed of the PA28α and PA28β subunits, which participates in the generation of antigenic peptides required for MHC class I Ag presentation. Previously, we have shown that expression of the PA28α subunit in mouse fibroblast B8 cells to a level similar to that obtained as a result of IFN-γ induction resulted in the enhanced MHC class I presentation of an influenza nucleoprotein and a murine CMV (MCMV)<sup>4</sup> immediate early (IE) protein pp89-derived epitope (7). In agreement with this and supporting the validity of in vitro digestion experiments, we also showed that in vitro processing of a larger synthetic polypeptide derived from the MCMV pp89 by 20S proteasome in the presence or absence of PA28 is able to support the generation of the pp89 MHC class I epitope with an efficiency comparable with that observed in vivo (8). In PA28β<sup>−/−</sup> mice, lacking the PA28α and PA28β subunits, CTL responses are impaired (9).

Peptides generated by the proteasome system in the cytoplasm are translocated into the lumen of the endoplasmic reticulum (ER), where they can associate with MHC class I molecules, a process that is mediated by specific IFN-γ-inducible peptide transporters named TAP. TAP binds cytoplasmic peptides with a broad, but not indistinguishable specificity, and translocates them into the ER in an ATP-dependent fashion (10). In general, TAP prefers peptides of about 9 aa, similar to MHC class I molecules, but can also translocate smaller and larger peptides albeit with lower efficiency (11, 12).

Up to now it is unclear which peptide fragments generated by the proteasome ultimately enter the ER. Are these by definition peptide fragments of the correct size for MHC class I binding, or are these in some cases precursors that require further trimming? In addition, the role of PA28 in this combined process is unresolved. Processing following proteasomal cleavage could take place in the cytosol. Beninga et al. (13) described a cytosolic IFN-γ-inducible leucine aminopeptidase that processed an epitope precursor in vitro. It remains to be shown whether this or other cytosolic peptides are involved in Ag processing in vivo. In addition, peptide competition experiments for TAP translocation suggest that N-terminal trimming may occur in the ER, but no ER peptidase was isolated to date (14, 15).

In this study, we have followed the degradation of a MCMV pp89 fragment by proteasomes in the presence or absence of PA28.

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4 Abbreviations used in this paper: MCMV, murine CMV; ER, endoplasmic reticulum; IE, immediate early; ms, mass spectrometry; RP, reversed phase.
For the first time, peptide generation by the proteasome system and TAP translocation have been experimentally linked. We show that PA28 is inducing the formation of an 11-mer epitope precursor fragment that is arriving in the ER. In contrast, the generated 9-mer epitope was not detected in the ER. Consequently, the 11-mer precursor represents the immunorelevant peptide product that requires N-terminal trimming in the ER for MHC class I binding.

Materials and Methods

Cell lines

Mouse B8 fibroblast cells (16) were cultured in IMDM supplemented with 10% FCS, 2 mM l-glutamine, and 10 U/100 μl penicillin/streptomycin. Induction with 20 U/ml mouse rIFN-γ (Boehringer Mannheim, Mannheim, Germany) was performed for 72 h. Human T3 cells (17) derived from TAP-deficient T2 cells, transfected with rat TAP1 and TAP2 and mouse EL4 cells were grown in HEPES-buffered RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 8% FCS. Human LCL 721 cells were grown in RPMI 1640, 8% FCS (18).

Proteasome purification

The 20S proteasomes were isolated from IFN-γ-induced (B8i) or noninduced (B8) mouse B8 fibroblast cells. About 6 × 10^7 cells were harvested with CaCl_2 /MgCl_2 -free medium and homogenized in 5 vol buffer A (10 mM HEPES, pH 7.2, 80 mM KCl, 5 mM MgCl_2 ) plus 0.1% Triton X-100. The cleared lysate was applied onto DEAE Sephacel (Pharmacia, Freiburg, Germany) and washed. Proteasomes were eluted with 500 mM KCl in buffer A and concentrated by ammonium sulfate precipitation between 40 and 70% saturation. This protein fraction was further separated by ultra-centrifugation through 10–40% sucrose gradients (Beckman SW40 rotor, 15.49 h at 38,000 rpm; Beckman, Fullerton, CA). The proteasome-containing fractions were pooled and applied to a Resource Q column (fast protein liquid chromatography; Pharmacia) and eluted with a gradient of 100–500 mM KCl in 10 mM HEPES, pH 7.2, 5 mM MgCl_2. Rechromatography of isolated proteasomes was performed by fast protein liquid chromatography on a MonoQ column (Pharmacia).

Expression and purification of rPA28

Recombinant PA28α and PA28β were expressed in Echerichia coli as GST-fusion proteins and purified, as described previously (19).

Peptide digests and HPLC separation

For digestion of a synthetic 25-mer peptide modified from the sequence of MCMV IE protein pp89, 5 μg peptide was dissolved in 100 μl HPLC buffer (20 mM HEPES, pH 7.8, 2 mM potassium acetate, 1 mM dithioerythrit) and incubated with 0.5 μl elastase (Boehringer Mannheim) at 37°C overnight. Samples were separated by TLC on Kieselgel 60 plates (Merck, Darmstadt, Germany) and dried and exposed to Kodak X-AR5 films or to a phosphor imager (BAS Reader 2000, TINA 2.0 software; Fuji, Tokyo, Japan).

Analysis of translocated peptides by ms

A total of 50 μg 25-mer peptide was digested with 4 μg B8 proteasome for 4 h at 37°C. The sample was translocated into 450 μl EL4 microsomes, as described above. Glycosylated peptides were bound to Con A-Sepharose and eluted with 250 mM methyl-α-mannopyranoside in 150 mM Tris, pH 7.4, 500 mM NaCl, 5 mM MgCl_2. Peptides were deglycosylated with 5 U N-glycosidase F (Boehringer Mannheim) at 37°C overnight. Samples were filtered (Membrex 4 CA, 0.2 μm; membranePure), adjusted to 1% acetic acid, 10% acetonitrile, and bound to C18 ziptips (Millipore, Eschborn, Germany). Peptides were eluted with 70% methanol, 1% acetic acid, and directly applied to the analysis by an ion trap mass spectrometer (LCQ; ThermoQuest, Bremen, Germany) equipped with an electrospray ion source. Ions with a mass/charge value of 662 Da (corresponding to the double-charged 11-mer peptide) were selected and fragmented by collision-induced dissociation.

Results

PA28 accelerates proteasomal epitope generation

To combine proteasomal degradation with translocation by TAP, we used a modified polypeptide originally derived from the MCMV IE protein pp89 (Fig. 1). An N-linked glycosylation site was introduced in the presented epitope by changing M_{10}P_{12} to N_{11}A_{13} to facilitate recovery of TAP-translocated peptides through...
the acquired oligosaccharides. Two D-amino acids at the extremities of the model peptide should prevent TAP-mediated translocation of the input 25-mer (21). To confirm that the modifications did not affect the degradation of the 25-mer model peptide, the peptide was incubated with 20S murine proteasomes in the absence or presence of rPA28α/β for various time points. As shown in Fig. 2, 20S proteasome is able to degrade the model peptide and generates 11- and 9-mer fragments containing the MHC class I epitope, as determined by ms. PA28 markedly accelerates the formation of the 11- and 9-mer fragments within the first hour of incubation. Note that the fragments are ultimately destroyed upon long incubations (24 h) in the presence of PA28, whereas free PA28 does not show any protease activity (Fig. 2H). A comparison of degradation products generated from wild-type or modified 25-mer peptide (Table I) shows that the modified 25-mer is a suitable substrate for the following studies. With few exceptions, the main products and the products of interest (9-mer/7–15 and 11-mer/5–15) were generated in both cases after 1 and 24 h.

Synthetic 11-mer precursor is translocated by TAP

TAP translocates peptides from the cytoplasm into the ER lumen in an ATP-dependent fashion. To test which of the substrates that include the presented epitope are potential substrates for TAP-dependent translocation, the 9-mer epitope, and the 11- and 25-mer input fragments were radiolabeled with 125I at tyrosine residues. The peptides were incubated with microsomes purified from cells expressing rat TAP1/2a in the absence or presence of ATP. In addition, microsomes from IFN-γ-induced B8 cells (B8i) were used in this assay. The translocated, and consequently glycosylated, fraction was isolated by Con A-Sepharose, and the bound

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Influence of PA28 on peptide generation by 20S proteasome. A–G, 25-mer was incubated with B8 proteasome in the absence (A, C, and E) or presence (B, D, and F) of recombinant PA28α and PA28β at 37°C. Aliquots were taken at the indicated time points: 0 h (G), 0.5 h (A and B), 1 h (C and D) and 24 h (E and F) of incubation. The products were separated by RP-HPLC and detected by electrospray ion source ms. H, 25-mer was incubated with PA28α/β for 1 h. The identity of dominant products is indicated.

![FIGURE 3](http://www.jimmunol.org/)

**FIGURE 3.** Transport efficiencies in in vitro translocation assays. Synthetic 9-, 11-, and 25-mer were incubated with isolated microsomes from T3 or B8i cells for 10 min at 37°C. Translocated peptides were isolated, and the Con A-Sepharose-bound radioactivity (percentage of translocated peptide) was determined in samples plus (●) or minus (□) ATP. T3 data are represented by filled boxes; B8i data by hatched boxes. The data represent the mean ± SD of three independent experiments.

### Table 1. Degradation products of wild-type and modified 25-mer peptide

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<td>24-h digest</td>
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*Wild-type and modified 25-mer peptides were incubated with purified B8 proteasome in the absence (–PA28) or presence (+PA28) of recombinant PA28 at 37°C for 1 and 24 h respectively. The products were separated by RP-HPLC and detected by ESI-ms.

b The peptides are ordered by estimated amount.

c The 9-mer is a minor degradation product.
peptides were quantitated (Fig. 3). Only the synthetic 11-mer peptide was efficiently translocated by TAP in the presence of ATP. In contrast, no transport was observed for the 9-mer epitope and the 25-mer input peptide. The same results were obtained with microsomes from murine (EL4 cells) or human cells (LCL 721 cells) (data not shown).

Combined proteasomal degradation and TAP-dependent translocation assays reveal translocation of the 11-mer, but not the 9-mer proteasomal degradation product

Efficient generation of an epitope does not necessarily imply that it is also efficiently appearing in the ER lumen and vice versa. Therefore, we decided to combine the proteolytic digest with the translocation system (Fig. 4). The modified pp89 25-mer was incubated with immunoproteasome (B8i) and rPA28α/β for 1 h. Sequentially, translocation assays were performed in the presence or absence of ATP. A simultaneous degradation and translocation assay could not be used, since isolated microsomes were not stable at 37°C for longer time periods even under isotonic conditions (data not shown).

To characterize the translocation products, the microsomes were washed and translocated peptides were recovered by Con A-Sepharose. After elution, the oligosaccharide chains were washed and translocated peptides were recovered by Con A-Sepharose. After deglycosylation by endoglycosidase H, one N-acetylglucosamine residue is left at the asparagine, which additionally influences the peptide properties. We observed the same two spots using the synthetic 11-mer in this translocation assay (Fig. 4, lane 2), which strongly suggests that, after proteasomal degradation of the 25-mer, only the 11-mer is transported. A third band in the second lane is unspecific since it is also found in the assay without ATP (lane 1). Assays with synthetic 9- and 25-mer showed no signals over background level (data not shown).

The 11-mer is actively translocated into isolated microsomes as it is generated

The 11-mer precursor peptide is more prominently generated in the presence of PA28, as determined by ms (data not shown). To test whether this peptide also enters the ER more efficiently, proteasomal degradation was coupled with translocation assays. The pp89 25-mer was incubated for up to 6 h with murine constitutive (B8) or immunoproteasome (B8i) in the presence or absence of rPA28α/β. The subsequent translocation into T3 microsomes was performed for 10 min in the presence or absence of ATP. Samples of the degradation products and the translocated peptides were separated by TLC.

The separation of the degradation products on TLC plates enabled us to detect even small amounts of radioactively labeled fragments (Fig. 6). The amount of the input peptide decreased, and several degradation products appeared with ongoing degradation. Surprisingly, not only the 11-mer, but also the 9-mer could be detected after 30 min of incubation with both proteasome subtypes B8 and B8i, respectively (Fig. 6A). It accumulated as the incubation time increased. PA28 enhanced 20S proteasome activity. In the presence of PA28, B8, and B8i proteasomes generated higher amounts of 9- and 11-mer at the corresponding time points. Using B8i proteasome plus PA28, the levels of 9- and 11-mer decreased during extended incubation time (6 h), indicating further degradation of

![FIGURE 4](image)

**FIGURE 4.** The 11-mer is selectively translocated from the degradation products. Synthetic 11-mer and products of a 1-h degradation with B8 proteasome and rPA28α/β were translocated into B8i microsomes. Translocation assays were performed in the presence (+) or absence (−) of ATP for 10 min at 37°C. Translocated peptides were isolated, deglycosylated, and separated on TLC plates. The radioactively labeled peptides were detected by a phosphor imager. Radiolabeled 9- and 11-mer could be detected by ms/ms sequencing analysis. The 25-mer input peptide was incubated with B8 proteasome for 4 h, and the degradation products were translocated subsequently into EL4 microsomes. The translocated peptides were isolated, deglycosylated, and directly applied to the analysis by an ion trap mass spectrometer equipped with an electrospray ion source. Ions with a mass/charge value of 662 Da were selected and fragmentated by collision-induced dissociation. Fragments, generated from the 11-mer by dissociation of the indicated amino acids, are labeled.
the products. However, the amount of the input peptide decreased slower in assays plus PA28. This result could be confirmed by ms, whereas it was not observed with the wild-type substrate. The diminished total turnover might be due to the D-amino acids at the N and C terminus.

When the set of generated peptides was translocated by TAP, only the 11-mer was actively translocated (Fig. 7). From the degradation samples taken between 30 min and 6 h (Fig. 6), translocation products could be determined in all translocation assays with ATP. They were separated into two bands, with the lower one being the most prominent. No translocation products were found in samples without ATP and at 0 min of degradation. PA28 enhanced peptide generation, which resulted in increased precursor translocation. After 6 h of incubation, the 11-mer was further degraded in the presence of PA28, and in consequence less 11-mer product was translocated. Apart from the 11-mer, no other translocated peptides could be detected. Due to our experimental approach, the 11-mer epitope precursor is trapped in the ER by the acquired carbohydrate chain. In vivo, however, it has to be(trimmed by an ER resident peptidase to allow efficient MHC class I binding. Therefore, these experiments show that the immunodominant epitope of the MCMV pp89 protein is generated as an N-terminally elongated precursor by the proteasome, and that only this 11-mer peptide is translocated by TAPs.

**Discussion**

The repertoire of MHC class I-presented peptides is not only conditioned by the MHC class I-binding properties, but also by the components involved in peptide processing and the selectivity of the peptide transporter. By combining a proteasome-dependent processing system and a TAP-dependent peptide translocation system, we have shown that a MCMV pp89-derived immunodominant epitope has to be generated as precursor peptide to allow efficient transport into the ER.

The proteasomal cleavage properties determine the peptide pool, which may be translocated by TAP. Recent data suggest that the majority of MHC class I-bound peptides is generated by the proteasome without need for additional processing (22). However, several MHC class I epitopes are generated by the proteasome as putative precursor peptides (14, 23).

For a steadily increasing number of epitopes, including pp89 (16, 24), it has been shown by us and others that, with regard to both quantitative as well as qualitative aspects, the in vitro processing experiments using purified 20S proteasomes reflect the in vivo situation with high fidelity (15, 16, 22, 25, 26). Using this experimental approach in combination with a direct translocation assay, this study shows that the MHC class I epitope of the MCMV pp89 protein is generated and translocated as a precursor peptide.

In our in vitro experiments using purified 20S proteasome and rPA28, pp89 9- and 11-mer peptides could be detected after 30 min. Prolonged incubation times such as 24 h resulted in the further degradation of products generated at earlier time points (Fig. 2). This contrasts with previous studies in which incubation times of several hours were chosen to ensure total substrate turnover (24, 27). Given that generated peptides were translocated forthwith into the ER and are presented by MHC class I molecules within 1 h after virus infection (28), only the products of a first round of degradation would most likely fulfill in vivo conditions. It is tempting to speculate that unknown factors might improve Ag presentation by peptide channeling from proteasome to TAP. Further investigations are on its way to address this challenging question.

The immunodominant 9-mer could hardly be detected in previous studies (24) or only in the presence of PA28 (8). As expected, the modified substrate shows a slightly different cleavage pattern than the wild-type 25-mer. In vivo and in vitro studies elucidated the influence of flanking residues on the usage of cleavage sites.
(29) (Kuckelkorn et al., unpublished results). The amino acid changes in the epitope sequence necessary for peptide recovery after translocation changed the nature of the products. Nevertheless, the peptides of interest were generated. In addition, the radioisotope labeling of the substrate facilitated the detection of the generated epitope also in the absence of PA28. Constitutive (B8) and immunoproteasome (B8i) produced increased amounts of 9- and 11-mer in the presence of PA28. The effect of PA28 was even stronger for the immunoproteasome where these products decreased after 6 h.

Remarkably, PA28 does not change the cleavage pattern qualitatively, but accelerates the generation of distinct products. The same products were generated as without PA28, but at earlier time points. The epitope and its precursor were most efficiently generated by the immunoproteasome in the presence of PA28. These results were confirmed with the unmodified pp89 25-mer peptide and in studies with a p53 epitope (Kuckelkorn et al., unpublished results).

In case of the murine leukemia virus MCF8 epitope competition experiments recently showed that the epitope itself does not block the import of a reporter peptide, whereas the corresponding precursor peptides prevent translocation (13, 14). However, the ability of a peptide to inhibit the translocation of a reporter peptide does not necessarily imply that it is translocated itself. Consequently, only direct translocation assays are suited to determine whether a peptide is translocated or not.

In a first approach, we tested the translocation efficiencies of the pp89 epitope, its putative 11-mer precursor, and the 25-mer substrate peptide in in vitro translocation assays. Only the 11-mer was actively translocated according to the length and sequence specificity of TAP. The sequence specificity with regard to the C-terminal residue of the peptide differs in various species. Mouse TAP and rat TAPI/2 prefer peptides with hydrophobic or aromatic C-terminal residues, whereas human TAP and rat TAPI/2 also translocate peptides with basic C termini (30, reviewed in Ref. 31). With a hydrophobic amino acid (leucine) at the C terminus, the 11-mer was translocated by murine, rat, and human TAP. However, the 9-mer was not translocated due to proline at position 2. A proline residue at the first three positions of the peptide negatively influences transport (32, 33). In the putative precursor, this residue is situated at position 4, and therefore without any influence on ER translocation. It is noteworthy that proline at position 2 and leucine at position 9 of the 9-mer correspond to the MHC class I allele H-2Ld anchor residues (34–36). In addition, phenylalanine at position 4 is the main TCR contact residue (37). The 25-mer was not translocated due to the 3-amino acids at the extremities, which prevent peptide translocation (21). Thus, to trigger a MHC class I-dependent CTL response, different requirements have to be conducted by the peptide at the level of TAP-dependent translocation and MHC class I binding.

To answer the question as to which degradation products are translocated by TAP, we combined for the first time proteasomal degradation with a TAP-dependent translocation system. A more detailed analysis of the translocated peptides by TLC identified the translocated degradation product as the 11-mer. The translocation products from synthetic 11-mer and the pp89 peptides generated by the proteasome separated identically on TLC plates. Most importantly, the nature of the translocated 11-mer was proven by ms. The results are in perfect agreement with the translocation efficiencies of the synthetic epitope and precursor.

The fact that translocated pp89 11-mer precursor accumulates in the ER and is not further trimmed in our approach (even in microsomes derived from B8i cells expressing the correspondent MHC class I haplotype) could be explained in that the carboxy-}

...MHC class I I-binders protect the peptide against further trimming. Translocation of peptides degraded up to 6 h revealed that the precursor is translocated at the same extent as it is generated in vitro. Even at longer incubation times, no 9-mer was translocated. A combination of three IFN-γ-inducible components involved in Ag presentation, immunoproteasome, PA28, and TAP promotes the best peptide supply into the ER.

The pp89 epitope is translocated into the ER as a precursor, whereas it is presented at the cell surface by MHC class I molecules as a 9-mer. This implies an additional processing step in the ER lumen. It is formally possible that a few 9-mer peptides enter the ER, despite the steric hindrance of a proline residue at position 2. However, there is no evidence that those would play a role in \( L^d \) presentation compared with a pathway that comprises very efficient translocation of a precursor peptide, followed by ER trimming. Nevertheless, cytosolic trimming of precursors may not be excluded for other peptides not carrying a proline residue at position 2.

The ER peptidase involved in MHC class I ligand trimming could not be identified until now, but has been predicted for some time. The variability of the N termini of peptides eluted from MHC class I molecules led to a model in which the N terminus may be processed while the C terminus is fixed by the MHC class I molecule as an anchor residue (38, 39). Experiments with signal sequence-linked peptides gave first experimental hints for ER trimming (40, 41). These peptides were imported independently of TAP into the ER, and C-terminal epitopes were liberated. However, an imprecise cleavage by the signal peptidase could not be excluded.

Recently, Paz et al. (42) detected indirectly proteolytic intermediates in the ER. The \( K^d \)-restricted peptide from OVA was expressed at the C terminus of a type II ER protein. Precursor peptides could be detected in the cytosolic and ER fractions, whereas the 8-mer epitope was only found in the ER in the presence of the corresponding MHC class I molecules. In the absence of the MHC class I molecules, a 9-mer could be detected as a proteolytic intermediate in the ER. These data suggest that peptide trimming may take place in the peptide-loading complex or in an MHC class I-bound stage.

We now show in a proteasome degradation system coupled to TAP translocation that a MHC class I peptide extended N-terminally by 2 aa is selected for ER import by TAP, whereas the correct sized fragment is excluded. These data illustrate the importance of a coupled degradation/translocation system. An ER resident amino peptidase as a further component of the Ag-processing machinery would mediate between the selectivity of TAP and the length and sequence specificity of the MHC class I molecule.

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References


