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Bipartite nuclear localization signal of matrin 3 is essential for vertebrate cells

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Abstract

Matrin 3, a nuclear matrix protein has potential (1) to withhold promiscuously edited RNAs within the nucleus in cooperation with $p54^{nrb}$ and PSF, (2) to mediate NMDA-induced neuronal death, and (3) to modulate promoter activity of genes proximal to matrix/scaffold attachment region (MAR/SAR). We identified a bipartite nuclear localization signal (NLS) of chicken matrin 3 (cmatr3) at residues 583–602. By expressing green fluorescent protein (GFP) fused to the NLS mutant in chicken DT40 cells, we showed an essential role of the NLS for cell proliferation. Furthermore, we showed that both clusters of basic amino acids and a linker of the bipartite NLS were essential and sufficient for the nuclear import of GFP. Exogenous cmatr3 rescued the HeLa cells where human matrin 3 was suppressed by RNA interference, but cmatr3 containing deletions at either of the basic amino acid clusters or the linker could not.

Keywords: Matrin 3; Nuclear import; Nuclear localization signal; DT40; RNAi; Phosphorylation

Matrin 3 is a 125-kDa protein localized in the nucleoplasm with a diffused fibrogranular pattern excluded from nucleoli [1]. Matrin 3 is among the nuclear phosphoproteins of HeLa cells [2] and its phosphorylation is assumed to cause neuronal death. Phosphorylation of matrin 3 by protein kinase A (PKA) in response to activation of the *N*-methyl-D-aspartic acid (NMDA) receptor led to degradation of matrin 3 [3]. Due to similarities in the sequences and the positions between matrin 3 and NP220 [4], a conserved stretch of N- and C-terminal regions are referred to as the matrin homologous domains MH1 and MH3, respectively. Two tandem RNA recognition motif (RRM) domains between MH1 and MH3 resemble those of polypyrimidine-tract binding protein PTB/hnRNP I [5].

Together with the transcription and splicing factors $p54^{nrb}$ and PTB-associated splicing factor (PSF), matrin 3 is involved in the complex that withholds promiscuously edited RNAs within the nucleus [6]. Matrin 3 (P130) can bind to highly repetitive sequences of matrix/scaffold attachment region (MAR/SAR) probably to modulate the activity of proximal promoters [7].

In this communication, we studied the nuclear import of matrin 3. Nuclear import of macromolecules occurs through the nuclear pore complex (NPC) embedded in the nuclear membrane. Proteins that undergo nuclear import contain a nuclear localization signal (NLS) [8]. Nuclear import of proteins containing classical NLS, which was first elucidated through characterization of NLS in SV40 large T antigen and nucleoplasmin, is mediated by a heterodimeric receptor complex composed of importin α and β. Importin α serves as an adaptor that binds classical NLSs while importin β mediates docking of the ternary transport complex to NPCs [9]. The NLS in SV40 large T antigen is a prototype of monopartite NLSs which consist of a single cluster of basic amino acids [10], while NLS in nucleoplasmin is a prototype of bipartite NLSs which consist of two clusters of basic amino acids separated by a linker of ~12 residues [11]. We identified a bipartite NLS in chicken matrin 3 (cmatr3) at residues 583–602 and showed that this NLS is required for the proliferation of chicken

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DT40 cells. We further showed that both basic amino acid clusters as well as the linker of this bipartite NLS are essential for exogenous cmatr3 to rescue HeLa cells in which expression of human matrin 3 (hmatr3) was suppressed.

Materials and methods

Construction of plasmids. To express GFP-tagged matrin 3 mutants, cDNA encoding GFP and cmatr3 were cloned into pCMV-Tag3B (Stratagene) or pCAGIP [12]. For detail, see supplementary material.

Cell culture and transfection. DT40 cells were cultured and transfected as described previously [13]. To express exogenous matrin 3, DT40 cells were transfected with pCAGIP-GFP-cmatr3 and selected in medium containing puromycin. Concentrations of drugs used for DT40 in this study were as follows: 1 mg/ml histidinol (Sigma), 30 μg/ml mycophenolic acid (Calbiochem), and 0.5 μg/ml puromycin (Sigma). HeLa and NIH3T3 cells were grown in DMEM (Nissui), supplemented with 10% fetal calf serum. Transfections were performed using FuGENE HD transfection reagent (Roche).

RNA interference. Small interfering RNA (siRNA) was synthesized using *in vitro* transcription as described [14]. The T7 primer oligonucleotide 5′-TAATACGACTCACTATA was annealed with template oligonucleotide containing an antisense sequence of transcribed RNA. The template oligonucleotides used for *in vitro* transcription were as follows for luciferase siRNA, 5′-TCTCTGATTTTTCTTGCGTCGAGTTCCCTATAGTGAGTCGTATTA and 5′-AAAACTCGACGCAAGAAAAATCAGAGACCCTATAGTGAGTCGTATTA; for hmatr3 siRNA, 5′-AGAAGATTCAGCACCTGGTTCTGTGCCCTATAGTGAGTCGTATTA and 5′-AACACAGAACCAGGTGCTGAATCTTCTCCCTATAGTGAGTCGTATTA. Transfection of siRNAs was performed with Lipofectamine RNAiMAX (Invitrogen).

Western blotting. Cells (1×10^6) were collected, washed with PBS and lysed in 50 μl sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) loading buffer. Following brief sonication and boiling, aliquots were subjected to 6% SDS–PAGE. After electrotransfer to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech), proteins were detected by anti-matrin 3 antibody or anti β-tubulin antibody (Santa Cruz Biotechnology) using ECL Western blotting detecting reagents (Amersham Pharmacia Biotech).

Immunofluorescence staining. Cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde/PBS for 15 min, permeabilized with 0.1% Triton X-100/PBS for 5 min, and blocked with 0.5% BSA/PBS for 30 min. After incubation with anti-matrin 3 antibody for 1 h, the cells were washed three times with 0.5% BSA/PBS, incubated with anti-rabbit Ig fluorescein isothiocyanate-conjugated antibody (Zymed) for 1 h, washed three times with PBS and mounted with Vectashield (Vector Laboratory). Nuclei were counterstained with 1 μg/ml Hoechst 33258.

Results and discussion

Identification of cmatr3 NLS

Potential NLSs were sought within the cmatr3 sequence (GenBank Accession No. BAB78469) using PSORT II program [15] and four candidate sequences were suggested (Fig. 1A). The sequences 145–148 and 872–887 were within

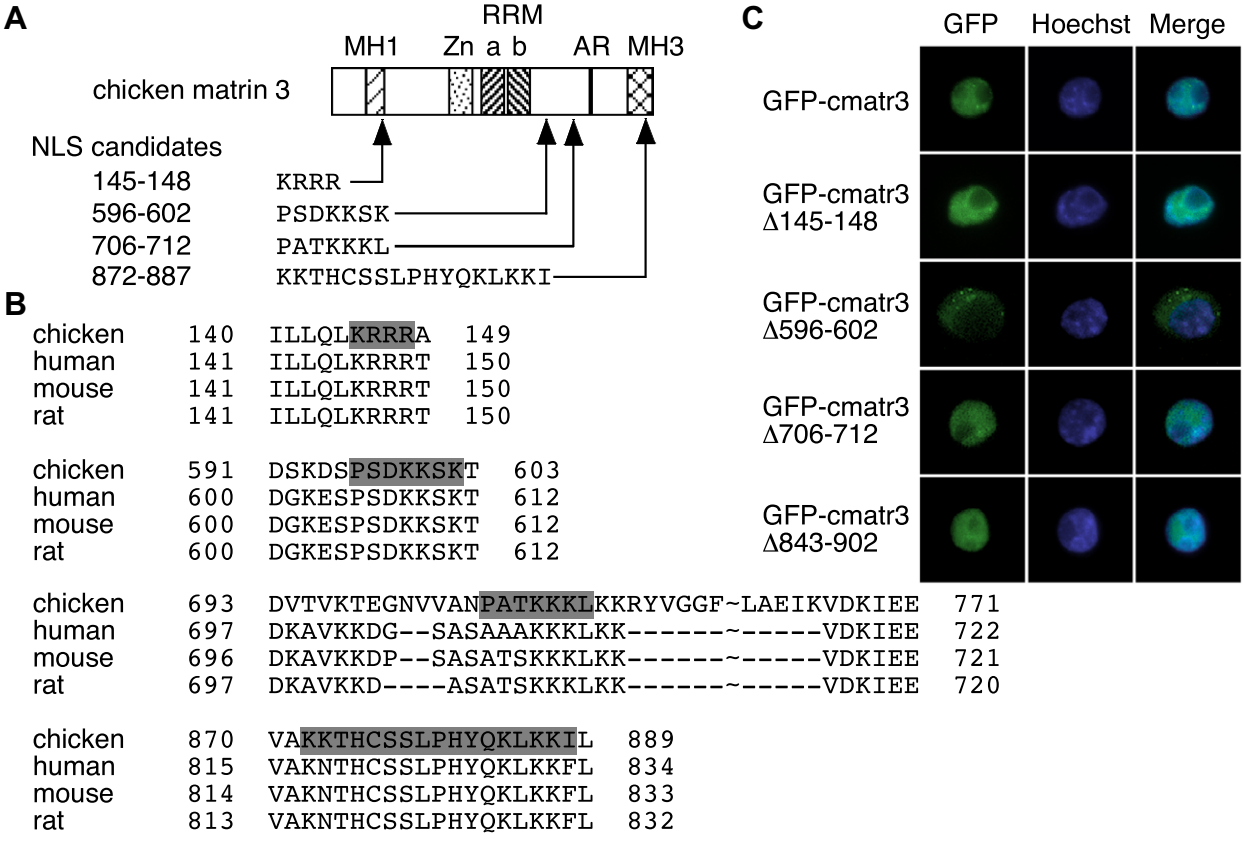

Fig. 1. Identification of cmatr3 NLS. (A) Schematic representation of cmatr3 and its predicted NLSs. (B) Alignment of predicted NLS of chicken (GenBank Accession No. BAB78469), human (AAH15031), mouse (AAH29070), and rat (AAB63955) matrin 3. The predicted NLSs are shaded. (C) Localization of cmatr3 deletion mutants. DT40 cells stably expressing GFP-cmatr3 or its deletion mutants were fixed and stained with Hoechst 33258 (blue).

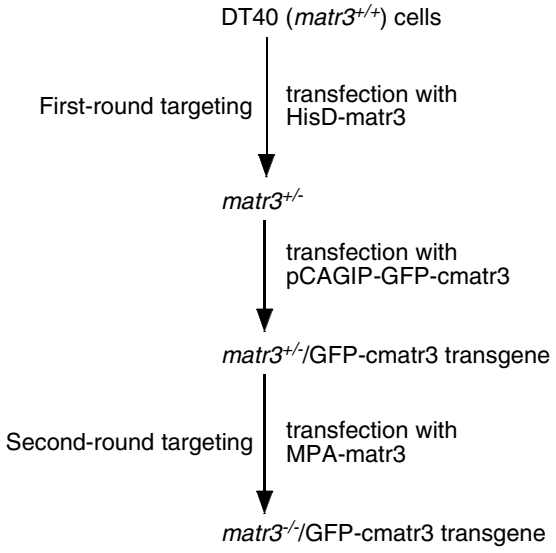

Fig. 2. Experimental strategy to rescue the lethality of $matr3^{-/-}$ DT40 cells.

the MH1 and MH3 domains, respectively. When compared with matrin 3 structures from several vertebrates, the four candidate sequences (Fig. 1B) were perfectly conserved except for the sequence 706–712, which showed minor diversity.

To identify the localization sequence responsible for nuclear import of cmatr3, four GFP-tagged cmatr3 mutants with deletions of each of the candidate sequences were constructed and expressed in chicken DT40 cells (Fig. 1C). Whereas Δ145–148, Δ706–712, and Δ843–902 as well as GFP-cmatr3 localized in nucleus, Δ596–602 remained in the cytoplasm, completely excluded from nucleus. Thus, we concluded that the NLS of cmatr3 contains the sequence 596–602 as an essential element.

NLS at residues 596–602 of cmatr3 is essential for the proliferation of chicken DT40 cells

DT40 is a chicken lymphoma cell line which allows targeted gene disruption at the cellular level [16]. In order to disrupt both alleles of the *matr3* gene, we constructed two targeting vectors containing either histidinol D (HisD) or mycophenolic acid (MPA) resistance gene (Supplemental Fig. 1A). While we could isolate several heterozygous knockout clones ($matr^{+/-}$), we could not obtain homozygous knockout clones ($matr3^{-/-}$), even after several attempts. We therefore conclude that a functional *matr3* allele is essential for proliferation of DT40 cells. Then, we tested which deletion mutants used in Fig. 1C can support the proliferation of $matr3^{-/-}$ DT40 clones. Fig. 2 shows the experimental procedure of the test. First we transfected the HisD-matr3 targeting vector into DT40 cells and isolated clones heterozygous for the *matr3* gene ($matr3^{+/-}$). Second, one of the $matr3^{+/-}$ clone was transfected with GFP-tagged matrin 3 expression vectors to obtain $matr3^{+/-}$ cells expressing GFP-cmatr3 ($matr3^{+/-}$/

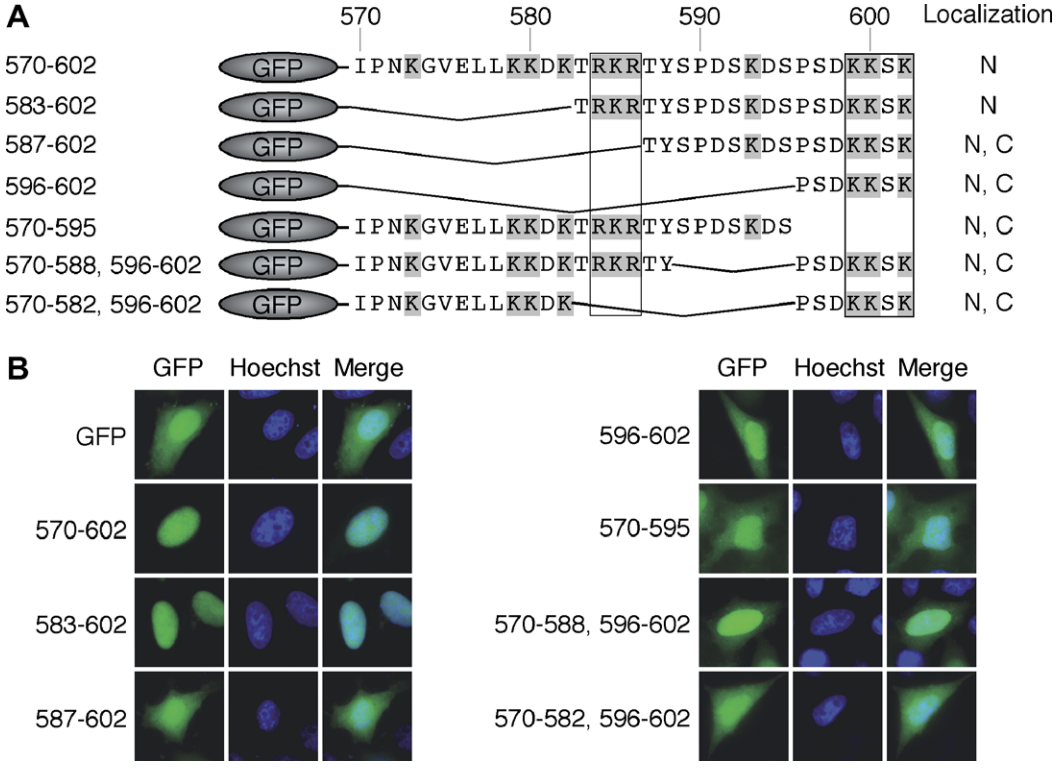

Fig. 3. Bipartite NLS of cmatr3 is sufficient for nuclear import of GFP. (A) Indicated sequence in cmatr3 NLS was fused to C-terminus of GFP. (B) HeLa cells were transfected with corresponding construct and localization of GFP was viewed by fluorescence microscopy after fixing and staining with Hoechst 33258 (blue). Subcellular localization is summarized at right margin of (A). N, nuclear; C, cytoplasmic. Basic amino acids are shaded. Boxes indicate clusters of basic amino acids in the bipartite NLS.

GFP-cmatr3). Third, we transfected the MPA-matr3 targeting vector into several *matr3*$^{+/-}$/GFP-cmatr3 clones to isolate *matr3*$^{-/-}$/GFP-cmatr3 clones. At every step of this test, the Southern blot pattern of *Eco*RI-digested genomic DNA from every clone was examined (Supplemental Fig. 1B) After second-round targeting, HisD/MPA-resistant clones showed the frequency of homologous targeting to be about 25% for most GFP-matrin 3 mutants. However, no homologous targeting was observed for GFP-cmatr3 Δ596–602, even after 96 clones were subjected to Southern blot analysis. These data indicated that NLS at residues 596–602 are essential for complementation of *matr3*.

All elements in bipartite cmatr3 NLS are essential and enough for nuclear import of GFP

In addition to ^{599}KKSK^{602}, cmatr3 has another cluster of basic amino acids, ^{584}RKR^{586}, interposed by a linker sequence of 12 residues (Fig. 3A). Since these elements compose a typical bipartite NLS, we prepared a series of expression vectors of GFPs fused to the deleted sequences listed in Fig. 3A, and expressed them in HeLa cells to examine which element is essential for the nuclear import of GFP.

Whereas GFP distributed in both nucleus and cytoplasm, GFP-cmatr3 (570–602) and GFP-cmatr3 (583–602) localized exclusively in nucleus (Fig. 3B). In contrast, neither GFP-cmatr3 (587–602) nor GFP-cmatr3 (596–602) localized predominantly in the nucleus, suggesting that both ^{584}RKR^{586} and the linker are essential for nuclear localization. As for cmatr3Δ596–602, absence of ^{599}KKSK^{602} in GFP-cmatr3 (570–595) resulted in failed nuclear import. Furthermore, GFP-cmatr3 (570–582, 596–602) accumulated in the nucleus to a lesser extent than did GFP-cmatr3 (570–588, 596–602), suggesting that ~12 residues of the linker sequence may allow each cluster to bind to different regions of importin α [17]. Altogether, these results showed that all elements of bipartite cmatr3 NLS are essential and sufficient for nuclear import of GFP.

All elements in bipartite cmatr3 NLS are essential for functional complementation of hmatr3-knocked down HeLa cells by cmatr3

We showed an essential role of elements in bipartite NLS of cmatr3 by using GFP as a foreign protein. Experiments with the expression of GFP-cmatr3 in HeLa cells

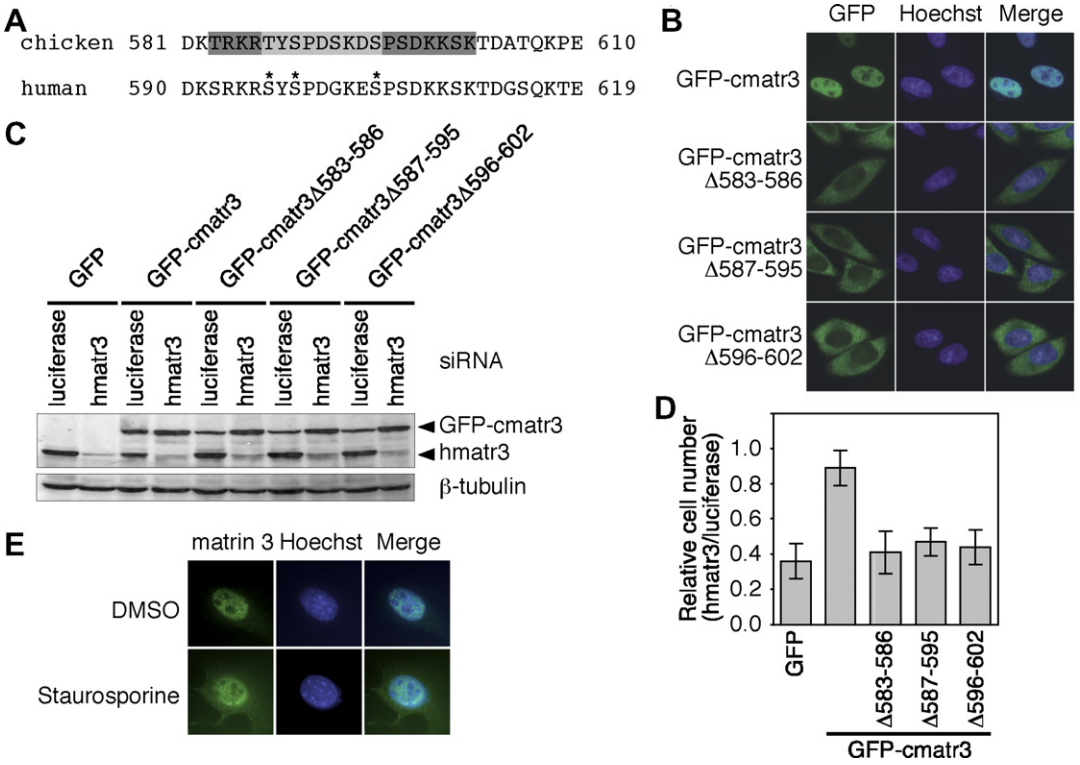

Fig. 4. Both basic amino acid clusters and the linker of cmatr3 NLS are essential for rescue of matr3-knocked down HeLa cells. (A) Sequence of cmatr3 surrounding bipartite NLS and its human counterpart are aligned. Shaded sequences were deleted from GFP-cmatr3 expressed in (B). Asterisks indicate phosphorylation sites in HeLa cells. (B) HeLa cells were transfected with indicated vectors, fixed and stained with Hoechst 33258 (blue). (C) HeLa cells were transfected with vectors for GFP, GFP-cmatr3, GFP-cmatr3Δ583–586, GFP-cmatr3Δ587–595 or GFP-cmatr3Δ596–602. One day later, the cells were transfected with luciferase or hmatr3 siRNA and incubated for 3 days in medium containing 1 μg/ml puromycin. Expression of GFP-cmatr3, hmatr3, and β-tubulin were examined by Western blot. (D) Three days after siRNA transfection, cells were stained with trypan blue and viable cells were counted. Cell number is expressed relative to that of luciferase siRNA transfection. Mean values of three cell countings are shown with standard deviation. (E) NIH3T3 cells were treated for 1 h with DMSO (vehicle) or 50 μM staurosporine, and immunostained with anti-matrin 3 antibody (green). Cell nuclei were stained with Hoechst 33258 (blue).

confirmed these conclusions. In contrast, while GFP localized both in nucleus and cytoplasm (Fig. 3B), GFP-cmatr3 fusion protein localized exclusively in nucleus and the deletion mutations introduced at either TRKR (GFP-cmatr3Δ583–586), the linker (GFP-cmatr3Δ587–595) or PSDKKSK (GFP-cmatr3Δ596–602) caused localization exclusively in cytoplasm (Fig. 4B).

To show the essential roles of all elements for localization of cmatr3 to its site of function, we tested which deletion mutant of GFP-cmatr3 can rescue HeLa cells under conditions where endogenous hmatr3 expression was suppressed. We first introduced GFP, GFP-cmatr3 or its deletion mutant and then introduced hmatr3 siRNA to knock down hmatr3 as confirmed in Fig. 4C by Western blot of cell lysate. When the growth of HeLa cells was monitored by vital cell counting, GFP-cmatr3 rescued the HeLa cells from growth suppression, but none of the deletion mutants were effective (Fig. 4D).

Phosphorylation in the vicinity of NLSs have shown to modulate NLS-dependent nuclear protein import [18]. Since 3 out of 4 phosphorylation sites identified for hmatr3 [2] are within the linker sequence of cmatr3 as shown in Fig. 4A. To examine whether phosphorylation of matrin 3 affects its localization, we treated NIH3T3 cells with general inhibitor of protein kinase, staurosporine and looked for changes in the distribution of matrin 3. We observed that inhibition of protein kinase with staurosporine led to an increse in the amount of matrin 3 in cytoplasm (Fig. 4E). Together these results suggest that nuclear distribution of matrin 3 is regulated by its phosphorylation status.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.191.

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