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Expression of Endogenous Retrovirus ev/J *gp85* Gene and Analysis of Its Immunoreactivity in Comparison with Exogenous Viral Protein*

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Abstract: The envelope gene *gp*85 of ev/J, a new family of endogenous avian retroviral sequences identified recently, has the most extensive nucleotide sequence identity ever described with ALV-J avian leukosis virus. This report described expression of ev/J envelope gene *gp*85 derived from commercial meat-type chicken using the Invitrogen Bac-to-Bac baculovirus expression system. The antigenicity and immunoreactivity of the recombinant endogenous *gp*85 gene product (SU) were analyzed by indirect immunofluorescence, Western blot, indirect and blocking Enzyme-Linked ImmunoSorbent Assay (ELISA) using JE9 monoclonal antibody (MAb) against the envelope protein of ALV-J (ADOL-4817), positive mouse antiserum against the ev/J *gp*85 SU and sera from chicken naturally infected with ALV-J. The results showed that the ev/J *gp*85 SU can bind specifically to JE9 MAb and antiserum from chicken naturally infected with ALV-J, and the binding reactivity between exogenous ALV-J *gp*85 SU and natural positive chicken serum against exogenous ALV-J can be blocked by positive mouse serum against the ev/J *gp*85 SU. It is concluded that recombinant endogenous *gp*85 gene product (SU) has close immunological relatedness to the envelope protein of exogenous ALV-J (ADOL-4817 and IMC_{10200} strain).

Key words: Avian endogenous retrovirus; ev/J; Envelope gene *gp*85; Expression; Immunoreactivity

Avian endogenous ev/J (or EAV-HP) is a member of the ancient endogenous avian retrovirus (EAV) family that are present in the *Gallus* species (2, 15). The ev/J family of endogenous retrovirus was recognized following the discovery of avian leukosis virus subgroup J (ALV-J) that is a new avian pathogen which emerged in late 1980s and which is the causative agent of myeloid leukosis (7). There are between 6 and 11 copies of ev/J proviruses in the chicken genome. These proviruses fall into six classes, all of which share a high degree of sequence identity and contain an internal deletion that removes all of the *pol* gene and various amounts of *gag* and *env* gene sequences. A single ev/J provirus termed ev/J clone 4-1, with a structure comprising a DNA copy of the subgenomic *env* transcript flanked by LTRs, has been

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found to harbour a full Env ORF (9, 10, 12, 13). Sequence comparison in the envelope gene has demonstrated that ALV-J shows over 97% nucleotide sequence identity to endogenous ev/J, but only 40% identity to those of the subgroup A to E viruses. The subgroup A to E envelope genes are between 80 and 85% identical (1). The higher sequence similarity of the ALV-J envelope to that of ev/J suggested that ALV-J might have emerged as a result of a recombination event between an unknown exogenous ALV and endogenous retrovirus ev/J.

Among the exogenous ALV encoded proteins, the envelope glycoprotein on the surface of retroviral particles, which is determined by sequence variations that cluster in the variable and hypervariable regions of *gp*85 gene, contains the determinants of subgroup specificity, neutralization and receptor binding, and plays a role in the induction of lymphoid and myeloid tumors (5, 14, 17). Endogenous ev/J might have potential impact on these key functions because of their high homology in envelope gene sequence. In addition to contributing to the emergence of ALV-J by recombination, embryonic expression of ev/J *env* is thought to be associated with the induction of immunological tolerance to further natural infection by ALV-J in meat-type chickens (11). To further understand the immunological relationship between exogenous ALV-J and endogenous ev/J SU glycoproteins, this study compared the immunological reactions of the protein encoded by endogenous ev/J *gp*85 gene expressed *in vitro* using the Invitrogen Bac-to-Bac system and the relevant protein of exogenous ALV-J. No immunological difference between exogenous ALV-J (ADOL-4817 and IMC_{10200} strain) and endogenous ev/J SU glycoprotein has been demonstrated *in vitro*.

MATERIALS AND METHODS

ev/J *gp*85 plasmid construction and expression

Escherichia coli DH5α™ and DH10Bac™ (Invitrogen™, Carlsbad, California, USA) competent cells were used as hosts in all DNA cloning and transposition procedures based on manufacturer's recommendations. The plasmids used in this work were the pGEM®-T Easy Vector Systems (Promega, Madison, Wisconsin, USA), pFastBac™1 and bacmids of the Bac-to-Bac® Baculovirus Expression Systems kit (Invitrogen™).

Endogenous ev/J *gp85* gene was amplified from commercial meat-type chicken and cloned into the pGEM-MTC*gp*85-like plasmid (18). pGEM-MTC*gp*85-like plasmid and FastBac™1 plasmid were digested by *Bam*H I and *pst* I, followed by purification using a DNA purification kit (Qiagen, Hilden, Germany). The pFastBac™1 vectors was linearized after digestion and ligated with the purified ev/J *gp85* fragment. The products of ligation were introduced into *E. coli* DH5α™ competent cells. Following selection, DNA of the selected colonies was extracted and analyzed by restriction digestion with *Bam*H I and *pst* I for the presence and correct orientation of the insert. Competent *E. coli* DH10Bac™ cells were transformed with recombinant DNA pFastBac-ev/J *gp85*. The resultant recombinant DNA of bacmid-pFastBac-ev/J *gp85* was isolated and analyzed by PCR using 5'-CTGGATCCATGGGAGTTCATCTATTGCAACACCCAG-3' (containing *Bam*H I) and 5'-TACTGCAGTTAGCGCCTGCTACGGTGGTGACC-3' (containing *Pst* I) as forward and reverse primers (16, 19). Sf9 cells were transfected with the recombinant DNA by using

CellFECTIN (Invitrogen™). After 72 h incubation post-transfection, supernatant and cotransfected cells were resuspended and centrifuged (12 000 r/min for 5 min, 4℃) and the supernatant containing recombinant baculoviruses was harvested. The cell pellet was resuspended in lysis buffer (50 mmol/L Tris-Cl pH6.8, 15 mmol/L NaCl, 5 mmol/L EDTA, 0.5% NP-40, 1mmol/L PMSF) for purifying the ev/J *gp85* protein. Sf9 cells infected with wild-type baculoviruses were used as negative control.

Indirect Immunofluorescence

Sf9 cells were infected with recombinant ev/J *gp*85 baculoviruses. Cells were washed with PBS, fixed with 80% acetone and used for immunofluorescence assays (IFA) with MAb JE9 (8) against the envelope protein of ALV-J (ADOL-4817) and goat anti-mouse IgG-conjugated fluorescein (1:200 in PBS, Sigma, Saint Louis, Missouri, USA).

Western blotting

Protein extracts from Sf9 cells infected with the recombinant virus were prepared in denaturing buffer and analyzed in a 12% sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE). The separated proteins on the gel were transferred to a nitrocellulose membrane in tank for immunodetection. The membrane was immersed overnight in blocking buffer (5% dry milk in TTBS), then incubated with a primary MAb JE9 (1: 300 in TTBS) for 45 min at 37 ℃. After rinsing 3 times with TTBS (20 mmol/L Tris, pH 7.5, 0.1 mol/L NaCl, 0.1 % Tween 20), the membrane was incubated with a secondary goat anti-mouse IgG-conjugated with peroxidase (Sigma, Saint Louis, Missouri, USA) (1: 8 000 in TTBS) for 45 min at 37℃. After rinsing for 20 min in TTBS, the protein bands were visualized using DAB substrate. Sf9 cells infected with wild-type baculoviruses were used as the negative control. Sf9 cells infected with rBac4817env (ADOL-4817 subgroup J ALV) was taken as the positive control.

Preparation of antisera

To prepare antibody against ev/J *gp*85 protein, mice were subcutaneously inoculated with 200 μL of the rBac-ev/J*gp*85-infected cell lysate premixed with the same volume of the Freund's complete adjuvant at day 0. Subsequently, on days 14 and 28, the same amounts of lysate with incomplete Freund's adjuvant were injected via the same route into the mice. Two weeks after the third dose, 100 μL of lysate was injected via lateral tail vein and an intraperitoneal route. Seven days after the last inoculation, mice were anesthetized and bled from the orbital venous plexus to death and the clotted blood was centrifuged at 4 000 r/min for 10 min. The serum was recovered and stored at -20℃.

Sera was collected in 2002 from 10 chickens at 75 weeks of age in an adult broiler breeder flock in Inner Mongolia, China that had a history of myeloid leukosis (ML) and from which strain IMC_{10200} ALV-J was isolated. This was used as primary antibody in the indirect ELISA.

Indirect and blocking ELISA

To investigate the immunoreactivity of endogenous ev/J *gp*85 protein, an indirect ELISA using either a commercial ALV-J ELISA kit coated with ALV-J antigen (IDEXX Laboratories Incorporation, Westbrook, Maine, USA) or microtiter plates coated with endogenous ev/J envelope *gp*85 protein and previously produced exogenous IMC_{10200} ALV-J *gp*85 protein (19) respectively were carried out. The protein was purified through a high-efficient Ni-NTA agarose

column (Invitrogen™). The encoding domain for the exogenous ALV-J *gp*85 SU protein expressed previously in an Invitrogen Bac-to-Bac baculovirus expression system (19) was obtained from an exogenous ALV-J (strain IMC_{10200}, a Chinese strain). All reagents were obtained from IDEXX Laboratories Incorporation, and tests were carried out according to the manufacturer's instructions. Briefly, 10 serum samples of chicken serum were diluted 50 fold (1: 50) with sample diluent prior to the assay. 100 μL of each diluted serum was then added into wells of the plate (triplicate per serum sample). Meanwhile, 100 μL of undiluted negative serum control (IDEXX) was added into well A1 and A2, and 100 μL of undiluted positive serum control (IDEXX) was added into well A3 and A4. The plate was incubated for 30 min at room temperature. Each well was then washed with 350 μL of PBS 3 times. Goat anti-chicken conjugate (100 μL) was dispensed into each well. The plate was incubated in room temperature for 30 minutes, followed by washing each well with 350 μL PBS 3 times. TMB solution (100 μL) was dispensed into each well. The plate was then incubated at room temperature for 15 minutes. Finally, 100 μL of stop solution was dispensed into each well to stop the reaction. The absorbance values were measured at wavelength 650nm and expressed as optical density (*OD*). ELISA *OD* readings were transformed to S/P values based on IDEXX's instructions: S/P = (X-N) / (P-N). (X: the average *OD* value of serum samples, P: the average *OD* value of positive serum control, N: the average *OD* value of negative serum control). The sample with S / P>0.6 was considered to be positive.

The immunogenicity of the endogenous ev/J *gp*85 protein was also evaluated by two blocking tests of indirect ELISA using microplates coated with two different exogenous viral antigens respectively as described above. After antigen coating overnight, the carbonate solution was discarded, and each well of the plates was washed by PBS buffer containing 0.05% Tween-20 three times and blocked with PBS buffer containing 10% skim milk powder at 37°C for 1h. The murine antisera against ev/J *gp*85 gene product were used as a blocking antibody and diluted in sample diluent (IDEXX) with 2-fold serial dilutions (1:5, 1:10, 1:20, 1:40 and 1:80) prior to the test. The sera from the non-immunized mice were used as negative controls with the same dilutions as the antisera. 100 μL of diluted mice serum was added into each well of the plate and the plate was then incubated at room temperature for 15 min. The rest steps of ELISA in this blocking test were performed as described above for the indirect ELISA. The blocking rate was calculated as follows: blocking rate (%) = (*OD* control – *OD* blocking-antibody) / *OD* control × 100.

RESULTS

Expression of ev/J *gp*85

After combination with pFastBac Donor Plasmids DNA, target DNA fragment from pGEM-MTC*gp*85-like vector was transinfected with *E.coli* DH5α and the positive clone was identified by *Bam*H I / *Pst* I digestion. The digestion revealed that the ev/J *gp*85 was ligated into the donor expression vector pFastBac™1. Following the transformation of DH-10Bac™ Escherichia coli component cells by pFast-Bac-ev/J *gp*85, recombinant bacmids rBacmid-pFastBac-ev/J *gp*85 were identified by blue/white

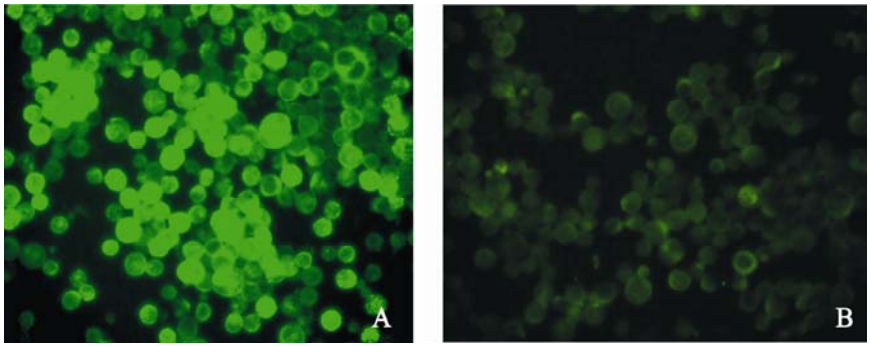

Fig.1. Indirect IFA staining of Sf9 cells infected with recombinant baculovirus encoding ev/J*gp*85. A: Sf9 cells infected with rBac-ev/J*gp*85. B: Sf9 cells with infected wild-type baculoviruses. Sf9 cells infected with rBac-ev/J*gp*85 showed stronger intensity of fluorescence than that of Sf9 cells infected wild-type baculoviruses.

selection and PCR analysis. PCR specifically amplified ev/J *gp*85 sequence from DNA samples of bacmid-pFastBac-ev/J *gp*85. Extracted recombinant DNA of rbacmid-pFastBac-ev/J*gp*85 was further used to co-transfect insect cells. The recombinant ev/J *gp*85 protein was characterized by IFA, Western-blot.

IFA staining of Sf9 cells infected with recombinant baculovirus

After transfection of Sf9 cells with recombinant baculovirus containing rBacmid-ev/J *gp*85 vector for 72 h, the expressed protein of the ev/J *gp*85 gene was detected by MAB JE9 against the envelope protein of ALV-J in an indirect immunofluorescence assay (Fig.1). This demonstrated the expression of ev/J *gp*85 gene in Sf9 cells. The Fig.2 also showed that the expressed protein of ev/J *gp*85 contained an epitope that was recognized specifically by monoclonal antibody JE9.

Western blotting

The expressed protein of ev/J *gp*85 was confirmed by western-blotting analysis using monoclonal antibody JE9. A stained band corresponding to 35 kDa was observed in an SDS-PAGE of lysated Sf9 cells infected with recombinant baculovirus containing rBac-ev/J *gp*85. No protein was detected from the lysate of Sf9 cells infected with wild-type baculoviruses (Fig.1). A stained band of 90~94 kDa protein (positive control) was detected from Sf9 cells infected with rBac4817 *env* vector.

Indirect ELISA of endogenous ev/J *gp*85 envelope protein

10 serum samples from an adult broiler breeder flock in Inner Mongolia, which had a history of myeloid leukosis (ML), were used as primary antibody in the indirect ELISA. The ELISA test plates

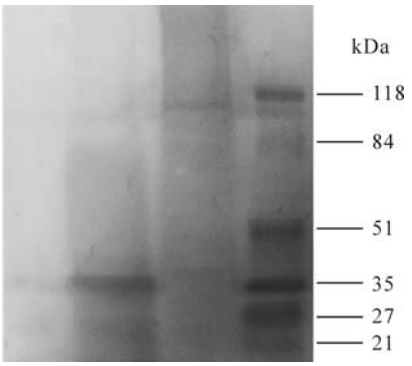

Fig. 2. Western blotting analysis of ev/J*gp*85 gene product expressed in Sf9 cells with JE9 MAb. 1, Lysate of Sf9 cells infected with wild-type baculoviruses; 2, Sf9 cells infected with rBac-ev/J*gp*85; 3, Sf9 cells infected with rBac4817; 4, Standard protein marker.

Table 1. Immunoreactivity analysis of ev/J SU protein by ELISA

Serum samples	119	220	201	202	203	204	205	206	207	208	P	N
IDEXX												
Average *OD*	0.184	0.283	0.316	0.163	0.195	0.266	0.220	0.362	0.390	0.275	0.409	0.163
S/P[a]	0.085	0.150	0.620	0.000	0.130	0.420	0.230	0.800	0.920	0.450		
ev/J *gp*85												
Average *OD*	0.167	0.293	0.309	0.170	0.201	0.266	0.230	0.372	0.407	0.282	0.398	0.160
S/P[a]	0.031	0.591	0.662	0.044	0.182	0.293	0.311	0.942	1.097	0.542		

[a]S/P=(X-N)/(P-N). X: the average *OD* value of samples, P: the average *OD* value of positive control, N: the average *OD* value of negative control. The S/P value over 0.6 is considered as positive. Sera as primary antibody were collected from an adult broiler breeder flock in Inner Mongolia, China that had a history of myeloid leukosis (ML).

coated with endogenous ev/J *gp*85 SU could detect the presence of the antibody against exogenous ALV-J. OD values were also compared with those from the IDEXX ALV-J ELISA kit (Table 1). Results in Table 1 showed that both endogenous protein encoded by ev/J *gp*85 gene and exogenous antigen of IDEXX kit could be recognized by positive antisera from the same chicken (201, 206 and 207) naturally infected with exogenous ALV-J. This data was similar to that using the plate coated with exogenous IMC_{10200} ALV-J *gp*85 SU protein with positive reactions of the same chicken sera (201, 206 and 207) (date not shown).

Blocking ELISA of the serum against endogenous ev/J *gp*85 envelope protein

To test immunogenicity of endogenous ev/J *gp*85 protein, the ELISA plate coated with exogenous IMC_{10200} ALV-J *gp*85 protein was used. As a blocking antibody, immunized mouse serum against endogenous ev/J *gp*85 protein was diluted in 2-fold serial dilutions, and performed to blocking analysis of ELISA. As a detection antibody, serum sample 207 was used and diluted 50 fold. Serum of non-immunized mice was taken as the control. The results showed that the serum against endogenous ev/J *gp*85 protein could block the ELISA reaction between exogenous IMC_{10200} ALV-J SU protein and natural positive chicken serum against exogenous ALV-J (Table 2). The blocking rates of endogenous ev/J *gp*85 protein antiserum were 54.66%~61.17% when compared with those using normal murine serum.

Furthermore, the immunized mouse serum was used

Table 2. Analysis of serum against ev/J *gp*85 gene product by blocking ELISA test[a]

Final serum dilution	5	10	20	40	80
IMC_{10200} ALV-J SU protein[b]					
mice antiserum	0.166	0.162	0.171	0.185	0.179
nornal mice serum	0.412	0.423	0.398	0.408	0.428
blocking rate	59.71	61.17	57.04	54.66	58.18
IDEXX ALV-J ELISA kit					
mice antiserum	0.166	0.173	0.177	0.171	0.182
nornal mice serum	0.416	0.399	0.398	0.396	0.391
blocking rate	60.10	56.64	55.52	56.53	53.45

[a]Serum sample 207 was used as a detection antibody. [b]ELISA microplates were coated with extrogenous IMC_{10200} ALV-J SU protein.

to perform blocking effect in the reaction between serum sample 207 and IDEXX exogenous ALV-J *gp*85 protein. Data obtained demonstrated that antiserum of endogenous ev/J *gp*85 protein could block the ELISA reaction between extrogenous ALV-J SU protein and natural positive chicken serum (Table 2).

DISCUSSION

Different structures of EAV-HP or ev/J proviruses have been identified in the chicken genome. Most of them in chickens show large deletions, including that of the entire *pol* gene, leaving the structural *gag* gene fused to the *env* sequences. While the deletion junctions in some of the proviruses give rise to in-frame gene fusions, most have single-base-pair insertions in or deletions from the *gag* region, preventing expression of any functional proteins (11). A single clone, designated ev/J clone 4-1, with the structure of a spliced *env* subgenomic transcript bounded by LTRs is the only chicken EAV-HP element described to date that possesses a complete *env* gene including the endoplasmic reticulum translocation signal coding sequence (9, 11). Based on exceptionally high sequence identity with ALV-J with respect to the *env* gene, it has been postulated that ev/J envelope could be responsible for the emergence of high-virulent virions via recombination. Such high sequence identity may have potential impact on adaptive immune responses in meat-type chickens to ALV-J infection via early embryonic expression.

In the present study, the glycoprotein of the ev/J *gp*85 encording domain was expressed *in vitro*, the sequence characteristics of which has been described earlier (18). This *gp*85 gene (GenBank accession no. AY375314) has 99.6% identity to the published ev/J 4.1 prototype *gp*85 gene cloned from DF-1 cells. Several other native lineages of chicken, obtained from broiler farms and Chinese native chicken breeds in China, were also examined for the endogenous ev/J element, which showed that endogenous ev/J *gp*85 genes in Chinese chickens had over 97% correspondence to the *gp*85 gene of ev/J4.1 (unpublished). Based on the observations in an ongoing study, no significant difference in the endogenous ev/J *gp*85 genes in broilers in various areas of P.R. China, has been observed (Yuying Yang, Yangtzeu University, Jingzhou, China). This differs from the high variations of exogenous strains from natural outbreaks (18). It is not known if and how identical endogenous sequence would affect the immunity of chicken to the exogenous strains. To investigate such immunological relatedness further, the present study focused on immunological characteristics of the *in vitro* expressed protein encoded by endogenous ev/J SU sequence. The indirect ELISA results (Table 1) showed that there was no significant difference between the *OD* values for endogenous and exogenous ev/J *gp*85 proteins when the same antiserum from chicken naturally infected with strain IMC_{10200} ALV-J was used, indicating that the endogenous ev/J *gp*85 SU could detect the presence of the antibody against exogenous ALV-J (IDEXX). Furthermore, the *gp*85 gene of strain IMC_{10200} ALV-J has 99.4% homology with the endogenous *gp*85 gene. The antiserum against endogenous *gp*85 gene encoded protein also neutralized the immunoreaction between exogenous gene encoded protein and murine antiserum in the blocking ELISA, indicating that the proteins encoded

by endogenous and exogenous *gp*85 gene share common antigenic epitope(s). This study also showed that the monoclonal antibody JE9 against the envelope protein of ALV-J (ADOL-4817) could bind to ev/J SU (8). This result provides the further support that there is a similar immunogenicity between the antigenic epitope(s) of endogenous ev/J and exogenous ALV-J *gp*85. The above may indicate that if expressed *in vivo*, the endogenous ev/J *gp*85 gene encoded protein may bind to the antibodies produced against natural infection. It is not known if the endogenous ev/J *gp*85 gene encoded protein could either up-or downregulate immune response to natural ALV-J infection.

It is not known if and how endogenous gene *gp*85 of ev/J functions *in vivo*. It is well known that the exogenous ALV-J isolates that show over 97% nucleotide sequence identity to endogenous ev/J predominantly induce myeloid leukosis (ML), a property thought to be associated with their tropism for the cells of the myeloid lineage. The oncogenicity of ALV-J was investigated by evaluating the viral envelope (5). The chicken Na+/H+ exchanger type 1 (chNHE1) has been recently identified as a functional receptor for pathogenic subgroup J avian leukosis virus, and the expression of chNHE1 in nonsusceptible cells lead to binding of Env J (the envelope protein of ALV-J) SU to these cells and conferred susceptibility to ALV-J envelope-mediated infection *in vitro* (4). For the endogenous counterpart, murine leukaemia virions functionally pseudotyped by ev/J 4.1 Rb can lead to a complete reciprocal interference with ALV-J envelopes, which indicates they share the same receptor (s) (6). Embryonic expression of EAV-HP *env* has been suggested as a factor associated with immunological tolerance induction in a proportion of ALV-J-infected meat-type chickens. Previous attempts to detect ev/J 4.1 *env* mRNA either from an ev0 chicken embryo cDNA library by RT-PCR or by Northern blot did not succeed (9). Whereas, an investigation showed that all EAV proviruses are transcriptionally active both in adult and embryonic tissues using three chicken EST databases (3). As previously suggested for ev/J, some earlier studies described the detection of the expression of ev/J in chicken. We also detected expression of ev/J in chicken embryo using RT-PCR (data not shown). However, the status of tolerance did not show any direct correlation with the presence of the intact EAV-HP sequence (11). These results encourage further investigations the function (s) of endogenous provirus ev/J SU *in vivo* and its effects on the host including on the immunoresponse, although no evidence was found to demonstrate the difference in their immunoactivity *in vitro*.

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