

# Disulfide bonds stabilize JC virus capsid-like structure by protecting calcium ions from chelation

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**Abstract** To investigate the role of disulfide bonds in the capsid structure, a recombinant JC virus-like particle (VLP) was used. The major capsid protein, VP1, of the JC virus was expressed in yeast cells. The yeast-expressed VP1 was self-assembled into a VLP. Disulfide bonds were found in the VLP which caused dimeric and trimeric VP1 linkages as demonstrated by non-reducing SDS-PAGE. The VLP remained intact when disulfide bonds were reduced by dithiothreitol. The VLP without disulfide bonds could be disassembled into capsomeres by EGTA alone, but those with disulfide bonds could not be disassembled by EGTA. Capsomeres were reassembled into VLPs in the presence of calcium ions. Capsomeres formed irregular aggregations instead of VLPs when treated with diamide to reconstitute the disulfide bonds. These results indicate that disulfide bonds play an important role in maintaining the integrity of the JC VLP by protecting calcium ions from chelation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:** JC virus; Assembly; Disulfide bond; Calcium ion

## 1. Introduction

Human JC virus is a small DNA virus which belongs to the Polyomaviridae. Severe immunosuppression may result in reactivation of the virus in human oligodendrocytes [1–3], and cause the fatal demyelinating disease, progressive multifocal leukoencephalopathy [4–6]. The virion of polyomavirus is composed of three structural proteins, major capsid protein (VP1), VP2, and VP3, as well as a viral minichromosome [7]. VP1 is the major structural protein of the capsid shell protein. The viral capsid consists of 72 pentameric capsomeres including 12 pentavalent and 60 hexavalent capsomeres [8]. Each capsomere contains five VP1 molecules. The minor structural proteins, VP2 and VP3, and the viral minichromosomes are encapsidated in the capsid particle [9,10].

Disulfide bonds are involved in maintaining capsid stability

of the polyomavirus. The importance of disulfide bonds in the polyomavirus capsid was first described by Walter and Depert [11]. Disulfide bonds have further been demonstrated to protect viral chromosomes from nuclease digestion in SV40 capsid [12]. More recently, disulfide linkages were illustrated between VP1 molecules in the capsid structure of SV40 and polyomavirus by X-ray crystallography [8,13–15]. Besides disulfide linkages, calcium ions were also found in polyomavirus and play an important role in viral assembly [16–18]. Although disulfide bonds and calcium ions have been demonstrated to be essential for maintaining the stability of the polyomavirus capsid, the relationship between disulfide bonds and calcium ions is still not clear. Both a reducing agent and a metal ion chelator are required to dissociate capsid particles to form the pentameric capsomeres. Either a reducing agent or a metal ion chelator alone can not disrupt the capsid particle [17,19]. Instead, capsomeres can be reassembled into a capsid-like structure in the presence of calcium ions alone without the involvement of disulfide bonds [18,20,21]. Therefore, the role of disulfide bonds in polyomavirus assembly needs to be further clarified. In the current study, we demonstrate that disulfide bonds play a role in protecting calcium ions from chelation in the JC virus-like particle (VLP).

## 2. Materials and methods

### 2.1. Expression of the JC virus VP1 protein in yeast cells

The VP1 gene of the JC virus (JCV) from a prokaryotic expressing plasmid, pGEXJCV1 [22], was cloned into the yeast-expressing plasmid, pFX7, to generate pFXJCV1 (a gift of K. Sasnauskas). The pFX7 plasmid contained GAL10 and PYK1 promoters for expressing the VP1 protein and FDH1 gene for formaldehyde selection. The pFXJCV1 plasmid was transfected into *Saccharomyces cerevisiae* (INVSC1) yeast cells according to the methods described by Gietz et al. [23]. INVSC1 yeast cells bearing the pFXJCV1 plasmid were grown in YEPD medium (1% yeast extract, 2% peptone, and 2% glucose) containing formaldehyde (final concentration 30 µl/100 ml). The yeast cells were cultured at 30°C with shaking for 16 h, after which galactose (final concentration 3%) was added to the culture for induction of JC virus VP1. Cells were harvested by centrifugation at 12000 rpm for 20 min after 24 h of induction. The cell pellet was resuspended in Tris buffer (10 mM Tris, pH 8.0, 1 mM EDTA, and 4.5 U/µl lyticase). After a 30-min incubation, glass beads were added to the suspension which was then vortexed for 10 min at room temperature. The cell suspension was frozen and thawed (–80°C to 37°C) twice, followed by incubation with DNase I (1 U/µl) and a protease inhibitor (1 mM PMSF) for 30 min. The cell lysate was centrifuged at 14000 rpm for 10 min, and the supernatant was collected for identification and purification of VP1 protein.

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**Abbreviations:** JCV, JC virus; VP1, major capsid protein; VLP, virus-like particle

## 2.2. Purification of the JC VLP

The supernatant of the yeast cell lysate containing VP1 protein was subjected to 20% sucrose cushion centrifugation at 35 000 rpm for 3 h in a SW41 Ti rotor (Beckman). The pellet was resuspended in Tris buffer and diluted to a density of 1.20 g/cm<sup>3</sup>, then layered onto a five-step CsCl velocity gradient consisting of 2 ml of 1.20, 1.25, 1.29, 1.32 and 1.35 g/cm<sup>3</sup> CsCl solutions [20,21]. After centrifugation at 35 000 rpm for 24 h, the solution was fractionated. Every fraction was analyzed by hemagglutination assay, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Western blot. Fractions with hemagglutination activity were collected and dialyzed against Tris buffer saline (TBS) (10 mM Tris–HCl, pH 7.4, 150 mM NaCl). The VLPs were concentrated by centrifugation at 40 000 rpm for 2.5 h.

## 2.3. Analysis of disulfide bonds in the JC VLP

8 µg of the purified VLPs was treated with SDS sample buffer without the reducing agent, β-mercaptoethanol. The mixture was resolved in a 7% SDS–PAGE after boiling for 10 min. VP1 protein was then transferred onto a nitrocellulose membrane after SDS–PAGE. The membrane was then blocked with 5% non-fat milk in Tris buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) and probed with rabbit anti-JCV VP1 serum at a dilution ratio of 1:3000. Goat anti-rabbit antibody, conjugated with biotin, and avidin conjugated with horseradish peroxidase (Vector, CA, USA) were used to detect VP1 protein.

## 2.4. Disassembly of the VLP

For disassembly, the purified VLPs were incubated with EGTA (10 mM) and/or dithiothreitol (DTT, 3 mM, final concentration) at room temperature (22°C) for 4 h [2,26]. After incubation, the mixtures were analyzed for hemagglutination activity and examined by electron microscopy.

## 2.5. Reassembly of the VLP

The disassembled capsomeres were dialyzed against reassembly buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% DMSO, and 0.5 mM CaCl<sub>2</sub>) at 4°C for 24 h [18]. The mixture was further analyzed for hemagglutination activity and examined by electron microscopy.

## 2.6. Reconstitution of disulfide bonds

8 µg of the purified VLPs or capsomeres was incubated with 0.5 mM diamide at room temperature for 2 h. After dialysis against Tris buffer, VP1 protein was resolved in a 7% SDS–PAGE without β-mercaptoethanol and was identified by Western blot. Morphologies of diamide-treated VLPs and capsomeres were observed by electron microscopy.

## 2.7. Sequential disassembly of the VLP

8 µg of the VLPs was incubated with 3 mM DTT or 10 mM EGTA

at room temperature for 4 h. After dialysis against Tris buffer at 4°C for 24 h, the mixtures were incubated with 10 mM EGTA or 3 mM DTT at room temperature for 4 h. Hemagglutination assay and non-reducing SDS–PAGE were performed at each step to monitor the integrity of the VLP and disulfide linkages.

## 2.8. Hemagglutination activity and hemagglutination inhibition activity

Human type-O red blood cells (0.5%) were used to determine hemagglutination activities of the JC VLPs in yeast cell lysate or in each reaction mixture. The detailed protocols for the hemagglutination assay were described previously [24]. JC virus-positive and JC virus-negative human sera [25] were used for the hemagglutination inhibition assay in this study.

## 2.9. Electron microscopy

Samples containing purified VLPs and capsomeres were mounted on formvar-coated grids and stained with 1% uranyl acetate [19–21]. Specimens were examined with a Joel 2000-CX electron microscope operated at 80 kV.

# 3. Results

## 3.1. Purification of the JC VLP

JC virus VP1 protein expressed in yeast cells was identified by Western blot using JC virus VP1 mono-specific antibody (data not shown). In addition, the yeast cell lysate containing VP1 protein showed strong hemagglutination activity (2<sup>14</sup>), and this activity was completely inhibited by JCV-positive human serum. These results support the idea that JC virus VP1 forms a VLP when expressed in yeast cells. Sucrose cushion and CsCl density gradient centrifugations were employed to further purify the VLPs. Fractions with strong hemagglutination activities were collected and analyzed by SDS–PAGE. The results showed that VP1 protein was purified to near homogeneity (data not shown). Electron microscopy also showed that VP1 was self-assembled into a VLP which measured approximately 45 ± 3 nm, *n* = 50, in diameter (Fig. 1B).

## 3.2. Analysis of disulfide linkages in the VLP

To demonstrate the presence of disulfide linkages in the VLP of JC virus, non-reducing SDS–PAGE was performed. Monomeric VP1 protein with a molecular weight of 42 kDa was found when the purified VLPs were resolved in a reducing SDS–PAGE with β-mercaptoethanol (Fig. 1A, lane 1). When

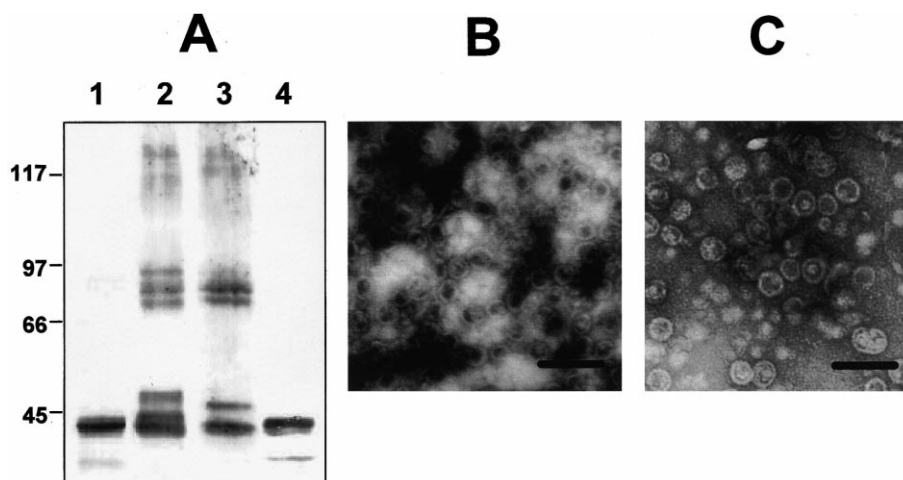


Fig. 1. Disulfide linkages in JC VLP. A: VLPs resolved by SDS–PAGE under a reducing (lane 1) or non-reducing (lane 2) condition. VLPs treated with diamide and resolved by SDS–PAGE under a non-reducing condition (lane 3). DTT-treated VLPs (lane 4) resolved by non-reducing SDS–PAGE. VP1 protein in A was identified by Western blot. Electron microscopy of native (B) and DTT-treated (C) VLPs. Bar = 100 nm.

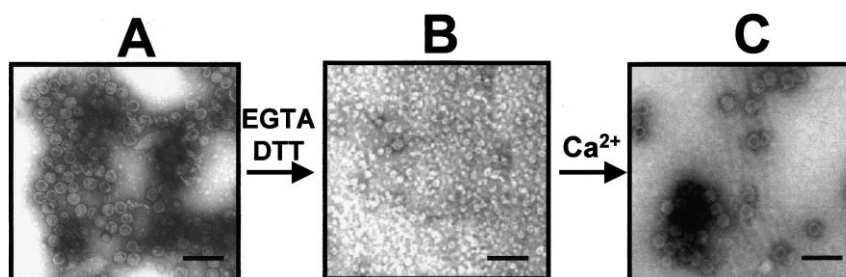


Fig. 2. Disassembly and reassembly of JC VLP. A: VLPs purified from yeast cells. B: Capsomeres disrupted from VLPs in the presence of both EGTA and DTT. C: Reassembled VLPs from capsomeres incubated with calcium ions. Bar = 100 nm.

the purified VLPs were resolved in SDS-PAGE without  $\beta$ -mercaptoethanol, four populations of VP1 with molecular weights of 42, 47, 80, and 120 kDa were found (Fig. 1A, lane 2). To rule out the possibility of host protein contamination of the purified VLPs, VP1 was identified by anti-JCV VP1 serum in immunoblots (Fig. 1A). These four populations were presumably two monomers (42 and 47 kDa), a dimer (80 kDa), and a trimer (120 kDa) of VP1 on the basis of their molecular weights. It is not clear what causes the 47-kDa VP1 formation in a non-reducing gel. Intramolecular disulfide linkages were presumably formed which caused the retarded migration, since the 47-kDa VP1 did not appear in the reducing SDS-PAGE. Monomeric VP1 was found in VLPs (Fig. 1A, lane 2) which might be attributable to the reduction of disulfide bonds during the purification process. To rule out this possibility, purified VLPs were treated with diamide to reconstitute the possible reduced disulfide bonds during the purification process [26]. A similar pattern was found in non-reducing SDS-PAGE for the diamide-treated VLPs (Fig. 1A, lane 3). Therefore, these results demonstrate that the VLPs contain monomeric, dimeric and trimeric VP1 molecules.

Previous experiments have shown that a reducing agent alone is unable to disassemble the polyomavirus capsid structure [17,19]. To further analyze if a reducing agent is able to break down disulfide bonds in the capsid structure, VLPs were treated with DTT. Disulfide bonds in VLPs were abolished resulting in only monomeric VP1 being present in the non-reducing gel (Fig. 1A, lane 4). In addition, the VLP without disulfide bonds retained its morphological integrity (Fig. 1C). The size of the VLPs treated with DTT ( $48 \pm 5$  nm,  $n = 50$ )

(Fig. 1C) was slightly larger than that of VLPs without DTT treatment ( $45 \pm 3$  nm,  $n = 50$ ) (Fig. 1B).

### 3.3. Disassembly and reassembly of the VLP

To determine the involvement of disulfide bonds and calcium ions in the JC capsid structure, purified VLPs (Fig. 2A) were treated with DTT and EGTA. The VLPs were completely dissociated to form pentameric capsomeres (Fig. 2B). Hemagglutination activity was also abolished when VLPs were disrupted into capsomeres. After removing the DTT and EGTA by dialysis, incubation of capsomeres with calcium ions resulted in reassembly into a VLP (Fig. 2C) with hemagglutination activity. These results are similar to previous reports [16,18,19,21] and indicate that disulfide bonds and calcium ions are involved in stabilizing the VLP, and that calcium ions but not disulfide bonds are required for capsid assembly.

### 3.4. Reconstitution of disulfide bonds

When treated with both DTT and EGTA, VLPs were disassembled to form capsomeres (Fig. 2B). To analyze whether disulfide bonds are present in capsomeres, capsomeres were resolved in non-reducing SDS-PAGE. The result showed that capsomeres do not contain intrapentameric disulfide bonds (Fig. 3A, lane 1). Calcium ions alone were able to reassemble pentameric capsomeres into VLPs as demonstrated in Fig. 2C. The effect of reconstituted disulfide bonds on capsomeres is not known. To understand this effect, capsomeres were treated with diamide to reconstitute the disulfide bonds. Non-reducing SDS-PAGE showed that both dimeric and trimeric forms of VP1 were re-formed (Fig. 3A, lane 2). The results indicate that the disulfide bonds between capsomeres were restored. Furthermore, capsomeres with reconstituted

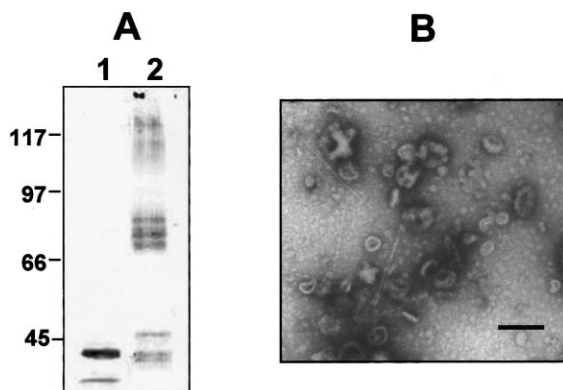


Fig. 3. Reconstitution of disulfide bonds between capsomeres. A: Capsomeres (lane 1) and diamide-treated capsomeres (lane 2) resolved by non-reducing SDS-PAGE. VP1 protein in A was identified by Western blot. B: Morphology of diamide-treated capsomeres. Bar = 100 nm.

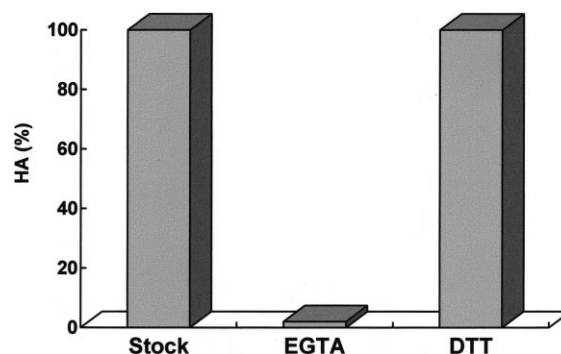


Fig. 4. Disassembly of calcium ions reassembled VLP. Reassembled VLPs are indicated as Stock. Reassembled VLPs were treated with EGTA or DTT alone.

disulfide bonds produced irregular aggregations, instead of VLPs, with diameters of between 20 and 70 nm as observed by electron microscopy (Fig. 3B).

### 3.5. Disassembly of reassembled VLP by chelator alone

Capsomeres were able to reassemble to form VLPs in the presence of calcium ions alone (Fig. 2C). The VLPs reassembled by calcium ions do not contain disulfide bonds (data not shown). Therefore, if disulfide bonds play a role in protecting calcium ions from chelation, treatment with a chelator alone should be capable of dissociating the reassembled VLPs. To verify this hypothesis, the reassembled VLPs were treated with either EGTA or DTT. The results showed that hemagglutination activity of the reassembled VLPs was abolished by treatment with EGTA (Fig. 4). Hemagglutination activity of the reassembled VLPs was not affected by treatment with DTT (Fig. 4). However, native VLPs could only be dissociated in the presence of both EGTA and DTT (Fig. 2B) instead of either one alone. These results are consistent with the idea that disulfide bonds play a role in protecting calcium ions from chelation.

### 3.6. Sequential disassembly of VLP

To further demonstrate the role of disulfide bonds in the capsid structure, VLPs were sequentially treated with EGTA then DTT or DTT then EGTA. After dialysis to remove the EGTA, the EGTA-treated VLPs were incubated with DTT. The VLPs remained intact and consequently retained their hemagglutination activity (Fig. 5, EGTA/DTT). In contrast, the VLPs treated with DTT first and then incubated with EGTA were disrupted into capsomeres and, therefore, lost their hemagglutination activity (Fig. 5, DTT/EGTA). As shown previously, diamide is able to reconstitute disulfide bonds between capsomeres (Fig. 3). Therefore, if the VLPs were to be sequentially treated with DTT and diamide, the particles should not be sensitive to EGTA dissociation. This hypothesis was verified by showing that the VLPs pretreated with DTT followed by treatment with diamide and EGTA retained their hemagglutination activity (Fig. 5, DTT/diamide/EGTA), indicating that the VLP remained intact. These results further demonstrate that disulfide bonds protect calcium ions from chelation and maintain the VLP structural integrity.

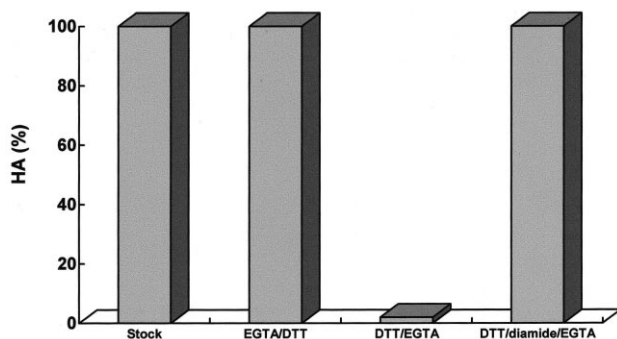


Fig. 5. Sequential disassembly of VLP. Purified VLPs (Stock) were sequentially treated with EGTA then DTT (EGTA/DTT); DTT then EGTA (DTT/EGTA); or DTT, diamide, then EGTA (DTT/diamide/EGTA).

## 4. Discussion

In this report, we showed that the JC VLP contained a trimeric, a dimeric, and two forms of monomeric VP1 (42 and 47 kDa). These findings indicate that there are two kinds of interpentameric disulfide linkages present in the VLP which cause dimeric and trimeric VP1 formation since intrapentameric disulfide bonds were not found. In addition, intra-VP1 disulfide bonds may also be present in the VP1 structure which produced a slower migration band (47 kDa). However, intra-VP1 disulfide linkages were not detected with X-ray crystallography of SV40 VP1 [8,14]. Therefore, formation of the 47-kDa JC virus VP1 in a non-reducing gel needs to be further investigated.

It is still not clear how the interpentameric disulfide bonds link together to form the dimer and trimer VP1 molecules. Refined X-ray crystallography of the SV40 capsid suggests that Cys-104 at the CD loop may be involved in disulfide bond formation with the same cysteine of a neighboring pentameric VP1 [14]. In addition, Cys-114 of polyoma VP1 (corresponding to Cys-104 of SV40) was found to covalently interact with Cys-19 at the N-terminus of the neighboring pentameric VP1 molecule [13]. These findings illustrate dimeric, but not trimeric, VP1 linkages. Recently, Cys-9, Cys-104, and Cys-207 of SV40 VP1 have been shown to be required for interpentameric disulfide linkages as demonstrated in a cell-free system [27] and baculovirus-expressing system [28]. These cysteines may be involved in dimeric and trimeric VP1 formation in order to maintain capsid stability [27]. However, Li et al. showed that mutation of each cysteine in the SV40 VP1 polypeptide did not affect viral infectivity [29]. When replacing Cys-254 with other long side chain amino acids, the infectivity was reduced. This effect is attributed to the longer side chains of the replaced amino acids possibly interfering with  $\text{Ca}^{2+}$  ion binding during capsid assembly [29]. Cys-41, 80, 97, 200, 247, and 260 in JCV VP1 are also conserved in SV40 VP1. The importance of each cysteine in the JC virus capsid structure needs to be further investigated.

The VLP of JC virus can be disrupted into pentameric capsomeres in the presence of both a reducing agent and a chelating agent but not either one alone. In addition, the pentameric capsomeres are able to reassemble into a VLP in the presence of calcium ions alone without restoring the disulfide linkages. These findings are similar to those of other reports [17,18,20,21] and indicate that calcium ions may play a crucial role in capsid assembly. However, the role disulfide bonds play in the VLP of JC virus was further defined in this study. After reducing the disulfide bonds, the DTT-pretreated VLP could be disassembled into pentameric capsomeres by EGTA alone. In addition, after incubating capsomeres with calcium ions, the reassembled VLP could also be dissociated into pentameric capsomeres by the chelator alone. These results are consistent with recent reports on murine polyomavirus and suggest that disulfide bonds might not be essential for the formation of the VLP but important to prevent capsid disassembly [30].

When the VLP was treated with DTT to abolish disulfide bonds, the structure remained intact, but it was enlarged. The enlarged capsid structure appeared to be sensitive to EGTA dissociation. These findings are similar to those of a previous report which showed that the SV40 virion treated with a reducing agent remained intact and was sensitive to nuclease

digestion of viral DNA [12]. Furthermore, although disulfide bonds between capsomeres were reconstituted after treatment with diamide, irregular aggregations were formed rather than VLPs. In contrast, capsomeres were able to reassemble into a VLP in the presence of calcium ions alone. Recently, Gharakhanian et al. also showed that disulfide linkages were found at a post-capsomeric stage during SV40 viral assembly [31]. Taken together, these findings imply that disulfide linkages are possibly not required for capsid assembly but may be essential for maintaining capsid structural integrity.

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