Disruption of Disulfide Bonds Is Responsible for Impaired Secretion in Human Complement Factor H Deficiency*

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Factor H, a secretory glycoprotein composed of 20 short consensus repeat modules, is an inhibitor of the complement system. Previous studies of inherited factor H deficiency revealed single amino acid substitutions at conserved cysteine residues, on one allele arginine for cysteine 518 (C518R) and on the other tyrosine for cysteine 941 (C941Y) (Ault, B. H., Schmidt, B. Z., Fowler, N. L., Kashtan, C. E., Ahmed, A. E., Vogt, B. A., and Colten, H. R. (1997) J. Biol. Chem. 272, 25168–25175). To ascertain if the phenotype, impaired secretion of factor H, is due to the C518R substitution or the C941Y substitution and to ascertain the mechanism by which secretion is impaired, we studied COS-1 and HepG2 cells transfected with wild type and several mutant factor H molecules. The results showed markedly impaired secretion of both C518R and C941Y factor H as well as that of factor H molecules bearing alanine or arginine substitutions at the Cys518–Cys546 disulfide bond (C518A, C546A, C546R, C518A-C546A). In each case, mutant factor H was retained in the endoplasmic reticulum and degraded relatively slowly as compared with most other mutant secretory and membrane proteins that are retained in the endoplasmic reticulum. These data indicate that impaired secretion of the naturally occurring C518R and C941Y mutant factor H proteins is due to disruption of framework-specific disulfide bonds in factor H short consensus repeat modules.

Complement factor H (2, 3), a regulatory protein of the alternative pathway of complement activation, inhibits the formation and accelerates the decay of the alternative pathway C3 convertase (C3bBb). It serves as a cofactor for the C3b-cleaving enzyme complement factor I (4). Factor H also has chemotactic activity for monocytes (5) and may perform functions related to interaction with extracellular matrix and leukocytes (6, 7).

Factor H is a single-chain serum glycoprotein with nine potential sites for asparagine-linked carbohydrates (8) and a total carbohydrate composition of 9–18% (3). It is a prototype of proteins with modular structure (9) consisting of a tandem array of homologous units, called short consensus repeats (SCRs), each about 60 amino acid residues in length (8). These structures have been recognized in 12 complement proteins and many noncomplement proteins including blood-clotting factor XIIIb, the α-chain of the interleukin-2 receptor, and cell adhesion molecules such as endothelial leukocyte adhesion molecule-1 and leukocyte adhesion molecule-1. The SCR motifs define a protein superfamily (10), characterized by conserved tyrosine, proline, and glycine residues and by the presence of four conserved cysteine residues, which form two disulfide bridges in a Cys3–Cys8 and Cys7–Cys12 fashion (11, 12). The 20 SCR modules of factor H are joined by short (3–8-amino acid) linkers, resulting in a shape that resembles a string of beads (13). The three-dimensional structure of both individual and paired SCR units has been determined and shows an autonomously folding structure with the two disulfide bridges far apart at the ends of a compact hydrophobic core (14).

Genetic deficiency of factor H has been described in domesticated animals (15) and in humans (16–23). Patients with homozygous factor H deficiency develop recurrent bacterial infections (including Nesseria sp.), vasculitis, and/or glomerulonephritis. The molecular basis for factor H deficiency has so far only been investigated in one patient, a child with glomerulonephritis (1). In skin fibroblasts from this patient there is an impairment in secretion of the large, 155-kDa form of factor H, while the small, 45-kDa form of factor H is secreted normally. The mutant factor H is retained in the endoplasmic reticulum (ER) of these cells. Analysis of the factor H cDNA sequence in the patient revealed single nucleotide substitutions on each allele: C518R substitution that affects the residue in the Cys2–Cys3 disulfide bond (11). The 20 SCR modules of factor H are joined by short (3–8-amino acid) linkers, resulting in a shape that resembles a string of beads (13). The three-dimensional structure of both individual and paired SCR units has been determined and shows an autonomously folding structure with the two disulfide bridges far apart at the ends of a compact hydrophobic core (14).

In the present study, we used site-directed mutagenesis to generate several specific mutant factor H molecules. The fate of these mutants was examined in transfected COS-1 and HepG2 cells to determine whether impaired secretion in factor H deficiency is caused by either the C518R substitution or the C941Y substitution or both and whether the mechanism by which these mutants are retained in the ER involves the specific amino acid substitutions or disruption of the associated disulfide bond.

EXPERIMENTAL PROCEDURES

Isolation of a Full-length Factor H cDNA Clone—A normal human liver cDNA library in Lambda Zap II (Stratagene, La Jolla, CA) provided by Dr. Rick Wetsel (24) was screened with a 1.4 kilobase pair cDNA probe (H-19, provided by Dr. Dennis Hourcade, Washington University, St. Louis, MO), which spans the nucleotide sequence from the first half of SCR 1 to the end of SCR 7 (25). A full-length clone (HL-2) was isolated and sequenced.

Site-directed Mutagenesis and Plasmid Constructs—Five mutants endoglycosidase H; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.4.

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The abbreviations used are: SCR, short consensus repeat; Endo H,
were constructed using the overlap polymerase chain reaction strategy (26). The following oligonucleotide pairs were used for mutagenesis: for mutant C518R TATGGGTACTRCAAGGAGGATGTTTGA; for mutant C518G TATGGGTACTAGGAGGATGTTTGA; and for mutant C518T TATGGGTACTCTCAGGAGGATGTTTGA. After Immunoprecipitin became unavailable, a preparation of pro-
molecules were subjected to fluorography on Kodak MR film (Eastman Kodak Co.) out methionine (Life Technologies) containing L-glutamine, HEPES, well plates on the first day post-transfection. 

Fibroblasts were not used after passage 12. Cells were plated in growth medium supplemented with penicillin/streptomycin. At timed intervals, medium was removed, and the cell monolayer was washed twice with warm Hanks' balanced salt solution and lysed by one freeze-thaw cycle in 100 mM sodium citrate (pH 5.5); 1% SDS, 100 mg/ml bovine serum albumin (fraction V; I CN Radiochemicals), N-glycosidase F (Boehringer Mannheim; EC 3.2.1.19). 

Immunofluorescence Microscopy—Transfected COS-1 cells were plated in growth medium on coverslips in 24-well plates 2 days after transfection. The coverslips were incubated overnight at 37 °C and then rinsed with PBS and fixed for 2 h at 4 °C in 4% paraformaldehyde in PBS. Coverslips were blocked with 50 mM NH4Cl in PBS, and the cells were permeabilized with 100% methanol at −20 °C for 7 min, rinsed with PBS (PBS containing 0.50 mM CaCl2 and 0.25 mM MgCl2), and blocked for 1 h at 4 °C with antibody dilution solution (PBS, containing 1% bovine serum albumin (Calbiochem) and 0.01% Tween 80 (Fierce)). 

The coverslips were then incubated overnight at 4°C with goat anti-human factor H IgG (INCSTAR, Stillwater, MN) and then washed four times with PBS, 0.1% Tween 20 (Sigma). After blocking, coverslips were incubated for 2 h with fluorescently labeled donkey anti-goat IgG (Binding Site) and then washed four times and mounted on microscope slides in Mowiol (Calbiochem, San Diego, CA) containing 2.5% 1,4-diazabicarboxyl [2,2.2] octane (Sigma). Slides were examined and photographed in a Zeiss Axioscope epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY). 

For the colocalization studies, goat anti-human factor H and rabbit antibody to the amino terminus of the ER protein calnexin (Stressgen, Victoria, Canada) were used. Texas Red-labeled donkey anti-rabbit IgG (Jackson Laboratories, West Grove, PA) was used as second label. For labeling the Golgi apparatus, Texas Red-labeled wheat germ agglutinin (Molecular Probes, Inc., Eugene, OR) was used at 2.5 μg/ml (31). Confocal images were taken using a ×63 immersion oil objective on a Zeiss epifluorescence microscope (Carl Zeiss) equipped with a Bio-Rad MRC laser confocal microscope adaptor. Images were processed using Adobe Photoshop software (version 4.0, Adobe, San Jose, CA). Nonimmune IgG and nonimmune serum were used in the first step of staining as controls and were found to not be significantly different from the unstained background. There was no fluorescence emission for fluorescein isothiocyanate at the filter setting for Texas Red and vice versa.

**RESULTS**

**Isolation of a Full-length Factor H cDNA Clone—**Several clones identified during screening of a normal human liver cDNA library were analyzed. Clone HL-2 contained the full-length factor H cDNA (3957 base pairs). The 5′-end of HL-2 is base 13 of the published factor H cDNA sequence, and the 3′-end of HL-2 extends 43 bases beyond the published sequence (Ref. 8; GenBank™ entry HSH.GB.PR, accession number Y00716). Sequence analysis demonstrated that HL-2 was identical to the published factor H cDNA sequence except for four nucleotide substitutions. One, A2089G, does not result in a change in the derived amino acid sequence (CAA and CAG both encode Gln(Glu)(34)). Two differences, C1277T and G2881T, result in amino acid substitutions that represent previously characterized polymorphic variants (H364Y and E918D) (32, 33). One difference, G1551C, which results in the presence of threonine rather than arginine at residue 475, has not been previously reported.

**Bioisotopic Labeling of Wild Type and Mutant Factor H in Transfected COS-1 Cells and HepG2 Cells—**First, we examined transfected COS-1 cells on days 1, 2, 3, and 5 after transfection for the optimal time of expression of wild type factor H (Fig. 1A). In each case, an ~165-kDa factor H polypeptide was present in the cells, and a slightly more slowly migrating, ~168-kDa factor H was in the extracellular fluid. The difference in the relative electrophoretic mobility of the intracellular and extracellular polypeptides is due to glycosylation, as deter-
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FIG. 1. Expression of wild type and mutant factor H in transfected COS-1 cells. A, COS-1 cells were transfected with wild type factor H cDNA using the DEAE-dextran method. Cells were labeled with methionine-free medium containing 250 μCi/ml TRAN35S for 5 h, on the first, second, third, or fifth day after transfection. The cell lysates (IC) and extracellular media (EC) were subjected to immunoprecipitation for factor H, and the immunoprecipitates were analyzed on a 7% SDS-PAGE gel. B, COS-1 cells were transiently transfected with vector alone (Vector), wild type factor H cDNA (WT), C518R mutant factor H cDNA, C518A mutant factor H cDNA, or C518A-C546A mutant factor H cDNA or were mock transfected (None), as indicated at the top. On the third day after transfection, cells were labeled and analyzed exactly as described for A. The electrophoretic migration of molecular mass markers (kDa) is indicated at the left of both panels.

FIG. 2. Expression of wild type and mutant factor H in transfected HepG2 cells. HepG2 cells were transfected using Lipofectin and then studied 36 h later. The cells were pulsed with cysteine- and methionine-free medium containing 250 μCi/ml TRAN35S for 7 h and analyzed exactly as described in the legend to Fig. 1. The migration of the more slowly migrating mature form of factor H only present in the cell lysates (IC) is indicated by an asterisk, and the migration of the more slowly migrating mature form of factor H but not present in cell lysates and extracellular medium (EC) of HepG2 cells transfected with wild type factor H is indicated by a double asterisk. The migration of molecular mass markers (kDa) is indicated at the left.

COS-1 cells (None). An ~168-kDa factor H polypeptide was present in the extracellular fluid of COS-1 cells expressing the wild type factor H construct. A slightly slower migrating polypeptide was seen in the extracellular medium of COS-1 cells transfected with C518R, C518A, and C518A-C546A factor H but in markedly lower amounts, indicating a block in secretion of the C518R, C518A, and C518A-C546A mutant factor H proteins. This decrease in secretion was also apparent at long (24-h) intervals of pulse radiolabeling (data not shown). A polypeptide of ~45 kDa was also present in cell lysates but is a nonspecific product of immunoprecipitation, because it was also present in untransfected COS-1 cells and in COS-1 cells transfected with vector alone, and its immunoprecipitation was not blocked by cold purified factor H (data not shown). In other experiments, there was also a marked decrease in secretion of factor H in COS-1 cells transfected with C546A, C546R, and C941Y mutant factor H constructs (data not shown).

We also compared wild type and mutant factor H expression in transfected HepG2 cells (Fig. 2). An ~156-kDa underglycosylated and ~168-kDa mature factor H polypeptide was detected in HepG2 cells transfected with wild type factor H. The ~156-kDa underglycosylated factor H polypeptide was predominantly present in HepG2 cells transfected with C518R, C546R, C518A, C546A, C518A-C546A, and C941Y mutant factor H. Factor H was not detected in mock-transfected HepG2 cells (None). An ~168-kDa factor H polypeptide was secreted into the extracellular medium only by HepG2 cells transfected with wild type factor H. Factor H could not be detected in the extracellular medium of HepG2 cells transfected with the mutant factor H constructs. Together with the results of transfected COS-1 cells, these data indicate that the impairment in secretion is neither cell- nor species-specific.

A comparison of the kinetics of secretion of wild type factor H with that of one mutant, C518R, in transfected COS-1 cells revealed (Fig. 3) loss of the wild type factor H from the cells between 1 and 3 h of the chase period, coincident with the appearance of the ~168-kDa factor H in the extracellular medium. Densitometric analysis of four separate experiments indicated that 95% of the initial factor H-specific radioactivity could be accounted for in cell lysates and extracellular medium taken together, and the half time for secretion was 2.5 h. In contrast, the C518R mutant factor H only began to disappear from the intracellular compartment between 6 and 12 h of the chase period, and none was detected in the extracellular fluid. The half time for its intracellular degradation was 7.77 h (n = 6).
The same pulse-chase protocol was used to investigate the fate of C941Y, C518A, C546R, C546A, and C518A-C546A mutant factor H molecules in transfected COS-1 cells. In each case, the mutant factor H molecule was retained in the cells, with none or a negligible amount detected in the extracellular fluid (data not shown).

In the experiments presented in Fig. 4, we used pulse-chase radiolabeling to compare the rate of intracellular degradation of each mutant factor H molecule in transfected COS-1 cells with that of mutant factor H expressed in the cell line from the compound heterozygous factor H-deficient individual. First, we compared the transfected COS-1 cells expressing the C518R and C941Y mutants to the factor H-deficient human fibroblast cell line (Fig. 4, A and B). The same ~165-kDa polypeptide was synthesized in each case. The rates of intracellular degradation were similar for C518R and C941Y mutant factor H molecules in the transfected COS-1 cells, but in each case factor H was somewhat more rapidly degraded than in the deficient fibroblast cell line. However, the degree of experimental variation and the difference in cell type make it difficult to conclude that this apparent difference in rate of degradation is physiologically significant. Next, we compared transfected COS-1 cells expressing the C518A, C546R, C546A, and C518A-C546A mutant factor H molecules with each other (Fig. 4, A and C). The rates of intracellular degradation were almost identical. Densitometry and phosphor imaging analysis showed the following results for the half-time of intracellular degradation: factor H-deficient fibroblasts, 11.5 ± 1.96 h (n = 6); C518R, 7.77 ± 0.92 h (n = 6); C941Y, 8.96 ± 1.29 h (n = 5); C518A, 7.46 ± 2.10 h (n = 5); C546R, 7.91 ± 0.88 h (n = 2); C546A, 7.97 ± 1.41 h (n = 4); C518A-C546A, 6.15 ± 1.04 h (n = 4).

Fate of Mutant Factor H in Transfected COS-1 Cells—All of the mutant factor H glycoproteins were sensitive to Endo H digestion even after 5 h of the chase period (Fig. 5A), consistent with retention in a pre-Golgi compartment. These results are comparable with similar experiments in control and factor H-deficient fibroblasts (Fig. 5B). All of the factor H retained in the factor H-deficient cells was sensitive to Endo H and N-glycosidase F, and none was secreted (right side of Fig. 5B). Although most of the factor H present in the cell lysates from control

![Fig. 3. Kinetics of secretion of wild type (top) and C518R mutant (bottom) factor H in transfected COS-1 cells. Three days after transfection, cells were pulsed with methionine-free medium containing 500 μCi/ml TRAN35S for 30 min and then chased in methionine-containing medium for the time periods indicated at the top. Cell lysates (IC) and extracellular media (EC) were analyzed exactly as described in the legend to Fig. 1. The migration of the molecular mass markers (kDa) is indicated at the left.](image)

![Fig. 4. Kinetics of intracellular degradation of different factor H mutants in transfected COS-1 cells and in fibroblasts from the factor H-deficient patient. A, cells were pulsed with cysteine- and methionine-free medium containing 500 μCi/ml TRAN35S for 90 min and then chased in complete medium for the time periods indicated at the top. Cells were analyzed exactly as described in the legend to Fig. 1. B and C, the results were also analyzed by densitometry or by phosphor imaging using the STORM system. The intensity of the signal for factor H at the time 0 of the chase period was arbitrarily considered 100%. The points in the graphs represent average values obtained from 2–6 experiments.](image)
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We used immunofluorescence to determine the intracellular localization of mutant factor H molecules in transfected COS-1 cells, control fibroblasts, and factor H-deficient fibroblasts. Cells were pulse-labeled with cysteine- and methionine-free medium containing 500 μCi/ml TRAN35S for 90 min and then chased for the time periods indicated at the top. Cell lysates (IC) and extracellular media (EC) were immunoprecipitated for factor H. A, the immunoprecipitates were then incubated in the absence or presence of Endo H (as indicated at the top of the gel) and then subjected to SDS-PAGE on a 6% gel. For C546R, the sample at 5 h of chase period that was not treated with Endo H was inadvertently not loaded. B, immunoprecipitates were incubated in the absence (−) or presence of Endo H (H) or N-glycosidase F (F) and then subjected to SDS-PAGE and fluorography.

![Diagram](image)

**FIG. 5.** Effect of Endo H and N-glycosidase F on factor H in transfected COS-1 cells, control fibroblasts, and factor H-deficient fibroblasts. Cells were pulse-labeled with cysteine- and methionine-free medium containing 500 μCi/ml TRAN35S for 90 min and then chased for the time periods indicated at the top. Cell lysates (IC) and extracellular media (EC) were immunoprecipitated for factor H. A, the immunoprecipitates were then incubated in the absence or presence of Endo H (as indicated at the top of the gel) and then subjected to SDS-PAGE on a 6% gel. For C546R, the sample at 5 h of chase period that was not treated with Endo H was inadvertently not loaded. B, immunoprecipitates were incubated in the absence (−) or presence of Endo H (H) or N-glycosidase F (F) and then subjected to SDS-PAGE and fluorography.

of factor H-specific immunofluorescent staining among COS-1 cells transfected with C518R, C546R, C518A, C546A, C518A-C546A, and C941Y mutant factor H constructs. In each case, there was a diffuse reticular staining of the cytoplasm especially prominent in the perinuclear region, which looked identical to the staining pattern of untransfected COS-1 cells with antibody to the ER-resident protein calnexin (data not shown). Double labeling experiments performed in COS-1 cells transfected with the C518R and the C941Y mutant factor H constructs showed colocalization of factor H and calnexin (Fig. 6). This colocalization was apparent in several focal planes. There was no colocalization of mutant factor H and a Golgi marker, wheat germ agglutinin (data not shown). Taken together with the Endo H experiments, these data indicate that all of the mutant factor H molecules are retained in the ER.

**DISCUSSION**

In a previous study, we showed that impaired secretion of factor H in a factor H-deficient child with glomerulonephritis was associated with single amino acid substitutions, C518R on one allele and C941Y on the other allele. In this study, we used transfected cells to express wild type factor H, mutant C518R factor H, and mutant C941Y factor H alone. The results showed impaired secretion of each of the mutants in both transfected COS-1 cells and transfected HepG2 cells, indicating that the defect in secretion is neither cell type- nor species-specific. Endoglycosidase H digestion and immunofluorescence studies indicate that each of these mutants is retained in the ER.

We then examined several possible mechanisms accounting for the impaired secretion of C518R and C941Y mutant factor H molecules. These mutations occur at the Cys5 position of the framework Cys4-Cys16 disulfide bridge in the ninth and 16th SCR module of factor H, respectively. Accordingly, using C518R as a model, we generated a series of mutant factor H molecules and transfected these to ascertain whether the impaired secretion was due to disruption of the disulfide bridge and/or a difference in size or charge of the substitution at residue 518. The results showed that the naturally occurring mutant, C518A, C546A, C546R (the latter two substituted at the Cys4 partner in the disulfide bridge) factor H proteins were retained and degraded in the same intracellular compartment. That C546A and C546R had the same fate as C518A and C518R in transfected cells established that disruption of the disulfide bridge is the major factor contributing to impaired secretion of the natural mutant factor H in the kindred previously described.

Similar results in studies of human lysozyme (34), influenza virus hemagglutinin (35), MHC class I molecules (36), and apolipoprotein B50 (37) support the conclusion that formation of specific disulfide bridges is critical for transport out of the ER to the cell surface or extracellular fluid. However, mutation of other disulfide bridges in lysozyme, influenza virus hemagglutinin, or apolipoprotein B50 had no effect on their secretion (34, 35, 37). In fact, in still other instances disruption of a disulfide bridge was associated with more rapid assembly and secretion (34, 38, 39). Finally, there are some examples where disruption of a disulfide bridge does not affect transport of the polypeptide when both cysteines have been mutated but a block in transport and ER retention is associated with an unpaired cysteine (40–42). To test for a potential role of unpaired cysteine residues in the secretory block of factor H deficiency, a mutant was constructed in which both Cys518 and Cys546 were substituted by alanine. The double mutant factor H C518A/C546A was also retained in the ER, making it highly unlikely that the generation of unpaired cysteines at residue position 546 or 518 was responsible for the block in secretion of the single mutant factor H molecules. Other human genetic deficiencies with substitu-
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FIG. 6. Double immunofluorescent labeling of factor H and calnexin in COS-1 cells transfected with factor H C518R (left column) or C941Y (right column). Images were taken in the same confocal plane using filters for green fluorescence corresponding to factor H (A and D) and red fluorescence corresponding to calnexin (B and E). The corresponding images (A with B and D with E) were overlaid to produce C and F, showing the colocalization of the green and red signal (producing orange). Colocalization was established in several other focal planes (data not shown).

proteins at critical cysteine residues and impaired secretion have been associated with the deficiency state (asparthylglucosaminuria (43), type 1 von Willebrand disease (44), factor XIIib deficiency (45), and protein C deficiency (46)), but in none has the mechanism been elucidated.

Several different mutant proteins are retained and degraded in the ER by a quality control apparatus that appears to involve the ubiquitin-dependent proteasomal degradation pathway (47, 48). The factor H mutants had a considerably longer half-life than most of the mutant secretory proteins (either naturally occurring or generated experimentally) that have been described in the literature. The half-time for degradation of the factor H mutants is on the order of 7–8 h, as compared with 1–3 h for other mutant proteins such as α1-antitrypsin Z (49), α1-antitrypsin S (50), mutant forms of complement C2 (51), complement C1 inhibitor (52), carboxypeptidase Y (53), factor XIIIb (45), and apolipoprotein B50 (37). Even when α1-antitrypsin Z is expressed in a genetic background characterized by a lag in ER degradation, the half-time for degradation is less (∼5–6 h) (49) than that of the mutant factor H molecules described here. Mutant membrane proteins and unassembled subunits of membrane-bound multisubunit proteins (e.g. CFTRΔF508 (54), the truncated form of ribophorin I (55), asialoglycoprotein receptor H2b (39), and T cell receptor α-chain (56)), which accumulate in the ER, are also rapidly degraded in that compartment. The physiologic degradation of the resident ER membrane protein, 3-hydroxy-3-methylglutaryl-CoA reductase, is also more rapid (57) than mutant factor H. The truncated T cell receptor α-subunit (generated experimentally by deletion of the transmembrane segment and cytoplasmic tail (58)) is the only other polypeptide that is retained in the ER as long as the mutant factor H. The possibility must therefore be considered that factor H is degraded by a novel mechanism or that the higher order structure of the mutant H confers unusual resistance to conventional degrading mechanisms. A mutant of factor XIIib (another member of the SCR-containing protein superfamily) substituted at one of the Cys residues of a Cys4–Cys4 disulfide bridge is degraded in the ER much more rapidly than any of the factor H mutants (45).

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