Regulated Cleavage of Sterol Regulatory Element Binding Proteins Requires Sequences on Both Sides of the Endoplasmic Reticulum Membrane

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Sterol regulatory element binding proteins (SREBP-1 and SREBP-2) are attached to the endoplasmic reticulum (ER) and nuclear envelope by a hairpin domain consisting of two transmembrane regions connected by a short luminal loop of ~30 hydrophilic amino acids. In sterol-depleted cells, a protease cleaves the protein in the region of the first transmembrane domain, releasing an NH$_2$-terminal fragment of ~500 amino acids that activates transcription of genes encoding the low density lipoprotein receptor and enzymes of cholesterol synthesis. In sterol-overloaded cells, proteolysis does not occur, and transcription is repressed. Through mutational analysis in transfected cells, we identify two segments of SREBPs that are required for proteolysis, one on either side of the ER membrane. An arginine in the luminal loop is essential. A tetrapeptide sequence (DRSR) on the cytosolic face adjacent to the first transmembrane domain is also required for maximal cleavage. Both of these elements are conserved in the human and hamster versions of SREBP-1 and SREBP-2. Sterol-mediated suppression of cleavage of SREBP-1 was found to be dependent on the extreme COOH-terminal region (residue 1034 to the COOH terminus), which exists in two forms as a result of alternative splicing. The form encoded by the "a" class exons (exons 18a and 19a) undergoes sterol-regulated cleavage. The form encoded by the "c" class exons (18c and 19c) is cleaved less efficiently and is not suppressed by sterols. These studies were made possible through use of a vector that achieves low level expression of epitope-tagged SREBPs under control of the relatively weak thymidine kinase promoter from herpes simplex virus. In contrast to SREBPs overproduced by high level expression vectors, the SREBPs produced at low levels were subject to the same regulated cleavage pattern as the endogenous SREBPs. These results indicate that sterol-regulated proteolysis of SREBPs is a complex process, requiring sequences on both sides of the ER membrane.

Cholesterol homeostasis is maintained by sterol-regulated cleavage of two membrane-bound transcription factors designated sterol regulatory element binding protein-1 and -2 (SREBP-1 and -2). These proteins, each nearly 1150 amino acids in length, are attached to the endoplasmic reticulum (ER) and nuclear envelope (1–3). When cells require cholesterol, a protease cleaves each of the SREBPs, releasing an NH$_2$-terminal fragment of ~500 amino acids that contains basic helix-loop-helix-leucine zipper and transcription activation domains. These fragments enter the nucleus and bind to sterol regulatory elements in the promoters of genes encoding the low density lipoprotein receptor, 3-hydroxy-3-methylglutaryl CoA synthase and possibly other enzymes of cholesterol and fatty acid biosynthesis (1–5). These actions lead to enhanced cholesterol uptake from plasma lipoproteins as well as enhanced cholesterol synthesis. When sterols overaccumulate in cells, proteolysis of the SREBPs is suppressed, the proteins remain bound to the ER, and transcription of the sterol-regulated genes declines.

In order to unravel the mechanism for sterol-regulated proteolysis, it is necessary first to understand the mechanism by which the SREBPs are attached to membranes. We recently used protease protection and N-linked glycosylation methods to demonstrate that SREBP-2 has a hairpin orientation (6). The NH$_2$-terminal and COOH-terminal segments of ~500 amino acids each face the cytosol. They are separated by a hairpin membrane attachment domain that consists of one transmembrane segment, a short 30-amino acid hydrophilic loop on the luminal side of the ER membrane, and a second transmembrane segment. The precise cleavage site and the domains within the SREBP that are required for proteolysis and for sterol-mediated suppression of proteolysis are unknown.

One way to answer these questions is through in vitro mutagenesis of cDNAs. We have not been able to use this method in the past because we were unable to observe sterol-mediated regulation when SREBPs were overexpressed in human embryonic kidney 293 cells as a result of transient transfection (2–4). Overexpression seemed to overwhelm the regulatory machinery, leading to aberrant and unregulated proteolysis. In the current studies, we have overcome this problem through the use of an expression vector containing a relatively weak promoter from the herpes simplex virus thymidine kinase (TK) gene (7). The promoter drives low level expression of SREBPs containing an epitope tag derived from a herpes simplex virus glycoprotein. The tag allows the products of the transfected cDNAs to be differentiated from the endogenous SREBPs. Under these conditions, cleavage of the epitope-tagged SREBPs is tightly regulated by sterols. Through mutagenesis studies, we identify two crucial segments in the SREBPs, one on either side of the membrane, that are required for proteolysis and a region in the COOH-terminal segment that is necessary in order for sterols to suppress this proteolysis.

EXPERIMENTAL PROCEDURES

Materials—HSV-Tag™ monoclonal antibody was purchased from Novagen. All other materials were obtained from sources as described previously (6).
Vector Construction of SREBPs—The following steps were used to construct a vector for human SREBP-2 in which expression is driven by the HSV TK promoter and the NH2 terminus of SREBP-2 is fused to two tandem copies of an 11-amino acid HSV epitope (see Fig. 1A): 1) We cloned the 3.2-kb Kpn–Sal fragment of human SREBP-2 (amino acids 316–1141) from pHX4 (3) into the Kpn–Sal site in pcDNA3 (Invitrogen) to create pSREBP-2. 2) The 5.1-kb pSREBP-2 fragment was digested with NotI and KpnI to remove the 0.7-kb cytomegalovirus (CMV) promoter in pcDNA3. 3) The 1.1-kb TK promoter from pTkβ (Clontech) was released with SalI and NotI (the SalI end was blunted with Klenow fragment). 4) A pair of complementary 124-base pair oligonucleotides (top strand), 5′-GGCCGCTAATACGACTCACTATAGGGAGCTAGTGCTAGCCATGCAGCCTGAACTCGCTCCAGAGGATCCGGAAGATATGCTG-3′, which contained 5′ NotI and 3′ KpnI cohesive ends, were annealed at 94–83 °C for 5 min and then 83–23 °C for 60 min. This oligonucleotide encoded, from 5′ to 3′, sequences for the following elements: NotI restriction site, T7 RNA polymerase promoter (for in vitro translation) followed by an inframe stop codon (TAG), SpeI and NotI restriction sites, initiator methionine codon in the context of a Kozak consensus (8), two tandem copies of the HSV epitope (QPELAPEDPED), from amino acids 265–275 of HSV envelope glycoprotein D and novel amino acids (IDTVTP) that correspond to the BspDI and KpnI restriction sites.

The above three fragments, namely the 7.9-kb NotI–KpnI fragment of the HSV TK promoter, the 316-1141 amino acid-encoding fragment of SREBP-2, the 1.1-kb SalI (blunted)–NotI fragment of the TK promoter, and the 124-base pair annealed oligonucleotids (5′ NotI and 3′ KpnI), were ligated together to create an intermediate plasmid, designated “triple ligation construct.” To clone the NH2 terminus of SREBP-2 into the triple ligation construct, the nucleotide sequence encoding amino acids 14–1141 of human SREBP-2 was amplified by polymerase chain reaction in a 100-μl reaction containing 200 ng of pHX4 (3), 2.5 units of pfu DNA polymerase, 5 nmol of dNTPs, and 20 pmol of each primer, which had a KpnI site at its 5′ end. The polymerase chain reaction was carried out at an initial temperature of 80 °C followed by 94 °C for 3 min and 20 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min for each cycle. The amplified product was digested with KpnI and cloned into the KpnI site of the triple ligation construct to create pTK-HSV- SREBP-2 (see Fig. 1A). This plasmid encodes an 1157-amino acid fusion protein of SREBP-2 (amino acids 14–1141) preceded by an initiator methionine, the two tandem copies of the HSV epitope, and six novel amino acids (IDTVTP) that correspond to the BspDI and KpnI restriction sites.

To construct an expression vector for human SREBP-1c, the following steps were used: 1) pSREBP-1c (2) was digested with EcoRI and XbaI to release a 1.2-kb XbaI fragment containing amino acids 2–1147. The amplified product was then digested with BspDI and EcoRI. pSREBP-1c-BspDI was digested with BspDI and EcoRI to remove the 4.2-kb SREBP-2 sequence but retain the HSV epitope sequence. The three fragments were ligated to generate pTK-HSV-BP1c. This plasmid encodes an 1071-amino acid fusion protein of SREBP-1c (amino acids 2–1147) preceded by an initiator methionine, the two tandem copies of the HSV epitope, and two novel amino acids (ID) corresponding to the BspDI restriction site.

To construct an expression vector for human SREBP-1b, pTK-HSV- BP1a was digested with SrfI and XbaI to release the 1.2-kb XbaI restriction fragment of SREBP-1a (amino acids 927–1147), after which this sequence was replaced with the 0.8-kb SrfI–XbaI fragment from pSREBP-1c (2) encoding amino acids 903–1047. The resulting plasmid is designated pTK-HSV-BP1b.

A control vector without any insert, designated pTK, was constructed by digesting pTK-HSV-BP2 with SpeI and XbaI to remove the 4.2-kb SREBP-2 sequence, followed by ligation. To construct a CMV promoter-driven expression vector for SREBP-2, 4.1-kb SpeI (blunted)-XbaI digested fragment encoding the HSV epitope tagged SREBP-1a fusion protein of 1171 amino acids (see above) was inserted into the EcoRV- XbaI sites of pcDNA3. This plasmid is designated pCMV-HSV-BP1a. All of the above plasmids were sequenced in the region of the construction junctions to confirm the DNA sequence.

The mutagenized construct was transformed into E. coli competent cells. The single-stranded, uracil-containing DNA was purified and used as template for site-directed mutagenesis. Oligonucleotides corresponding to the bottom strand of the SREBP sequences were synthesized, phosphorylated with T4 polynucleotide kinase, and annealed to the template under the following conditions: 94–83 °C for 5 min and then 83–23 °C for 60 min. The mutagenized strand was synthesized with T7 DNA polymerase, and the reactions were used to transform DH5α competent cells. Each mutant was sequenced to confirm the mutation. The multiple mutant clones were cut out at convenient restriction sites, cloned into the corresponding sites of the parental plasmid, and sequenced for confirmation. For the mutants that were not cut out and substituted into the parental plasmid, the mutation was confirmed by sequencing the relevant region, and at least two independent clones of the mutant plasmid were independently transfected into 293 cells to confirm the results.

Cell Culture, Transfection, and Cell Fractionation—Monolayers of human embryonic kidney 293 cells were transfected with wild-type or mutant plasmids as described (6). The cells were incubated for 20 h with 50 μM compactin and 50 μM sodium mevalonate in the absence or the presence of sterols as indicated in the legends. Thereafter, the cells received 20 μM lovastatin (Bristol-Myers) and 1 μM of cholesterol for 4 h. The transfected cells were then harvested and washed with phosphate-buffered saline containing 0.05% (v/v) Tween 20, 5% (v/v) nonfat dry milk, and 5% (v/v) heat-inactivated fetal calf serum. Immunodetection was carried out with Enhanced Chemiluminescent Western Blotting Detection Kit (Amersham Corp.) according to the manufacturer’s instructions except that the nitrocellulose was blocked in phosphate-buffered saline containing 0.5% (v/v) Tween 20, 5% (v/v) nonfat dry milk, and 5% (v/v) heat-inactivated fetal calf serum. Immunodetection of SREBPs was carried out with 0.5 μg/ml of HSV-Tag monoclonal antibody (6). Gels were calibrated with prestained molecular weight markers (New England Biolabs). The filters were exposed to Reflection film (DuPont NEN) at room temperature for the indicated times.

RESULTS

Fig. 1A shows the TK-driven vector that was used to express SREBP-2 in these studies. The NH2 terminus of the encoded protein contains a 22-amino acid epitope tag derived from the HSV envelope glycoprotein D (HSV tag). Expression is driven by the HSV TK promoter, and the polyadenylation signal is derived from the bovine growth hormone gene. A similar expression vector was constructed for SREBP-1a as described under “Experimental Procedures.” Fig. 1B shows the topology of the SREBPs and indicates the location of the two segments...
that are required for proteolysis: the DRSR immediately external to the first transmembrane sequence and the Arg in the lumenal loop.

Fig. 2 compares immunoblots of nuclear extracts and crude membrane pellets from human embryonic kidney 293 cells that were transfected with plasmids encoding HSV-tagged SREBP-1a under control of the strong CMV promoter or the weak TK promoter. The CMV vector gave extremely high level overexpression of the membrane-bound form of SREBP-1a, even when only 0.5 μg of plasmid was used (lanes 13–16 in Fig. 2B). The immunoblots showed multiple overlapping bands, indicating extensive proteolysis of the overexpressed protein. The amounts of the precursor and the prolytic fragments were similar when the cells were sterol-depleted by incubation with the cholesterol synthesis inhibitor compactin (−sterols) or when they were sterol-overloaded by incubation with 25-hydroxycholesterol and cholesterol in the presence of compactin (+sterols). Nuclear extracts of cells transfected with the CMV-driven vector contained relatively large amounts of the mature nuclear form of tagged SREBP-1a, but the amount was not affected by sterols (lanes 5–8 in Fig. 2A).

When the TK promoter was used, the amount of membrane-bound precursor was much lower than observed with the CMV promoter, and the smear of membrane-bound proteolytic fragments was not observed (Fig. 2, compare lanes 11–12 with lanes 13–16). The nuclei of cells expressing the TK-driven protein showed abundant mature form of SREBP-1a in the absence of sterols and a disappearance of this protein in the presence of sterols (Fig. 2, lanes 3 and 4). This behavior reflects that of endogenous SREBP-1a in untransfected cells (1, 13).

Fig. 3A shows the effect of different concentrations of 25-hydroxycholesterol on the amount of the mature nuclear form of tagged SREBP-2 produced by the TK vector. The protein disappeared at a sterol concentration between 0.3 and 1 μg/ml (Fig. 3A), which is similar to that previously observed for the endogenous protein (1, 13). The sterol had no effect on the amount of the membrane-bound precursor (Fig. 3B).

In an initial attempt to localize sequences in SREBP-1a that are required for sterol-regulated proteolysis, we created a series of point mutations in the linker region between the basic helix-loop-helix leucine zipper region and the first transmembrane domain, which is the approximate region at which cleavage takes place (4, 13–15). We previously identified an apoptosis-related cysteine protease that cleaves SREBP-1a at Asp460 and SREBP-2 at Asp468 (15). This protease, designated CPP32, becomes activated only during apoptosis, and it is not regulated by sterols. Changing the Asp at the cleavage site to Ala abrogates cleavage by CPP32 in vitro (15). However, changing these residues in SREBP-1a (Fig. 4B, lanes 5 and 6) or SREBP-2 (Fig. 4C, lanes 15 and 16) did not affect cleavage by the sterol-regulated protease in transfected 293 cells. These results provide further evidence that CPP32 is not responsible for sterol-regulated proteolysis.

SREBP-1a and -2 contain a conserved sequence DRSR that marks the boundary of the first transmembrane domain (Figs. 1B and 4A). The following residue, leucine (SREBP-1) or isoleucine (SREBP-2), initiates the hydrophobic sequence of the first transmembrane domain (6). We eliminated the DRSR by changing it to AS in both SREBP-1a and -2. We used AS because its coding sequence is a site for the restriction enzyme.
subjected to SDS-PAGE and immunoblot analysis with 0.5
g/ml 25-hydroxycholesterol plus 10 
μg/ml cholesterol (sterols) as indicated. On day 3, the cells were harvested and fractionated as described under “Experimental Procedures.” All aliquots of 40 μg of protein (B) or 60 μg (C) from nuclear extracts (upper panels) and aliquots of 60 (B) or 80 μg (C) from membranes (lower panels) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml HSV-Tag antibody. The filters in B and C were exposed to film for 30 s and 1 min, respectively. N and P denote the NH$_2$-terminal mature and precursor forms of SREBPs, respectively.

We next turned our attention to the short hydrophilic loop that lies on the lumenal side of the ER membrane between the two transmembrane regions (6). This loop contains a conserved Arg (residue 527 of SREBP-1a and 519 of SREBP-2) (Figs. 1B and 6A). Replacement of this Arg with an Ala abolished cleavage of both proteins (Fig. 6, B, lanes 5 and 6, and C, lanes 11 and 12). Changing the Arg to a Lys in SREBP-2 preserved the cleavage (lanes 13 and 14). This latter mutation was not made in SREBP-1a.

We previously showed that SREBP-1 can exist in several

**Fig. 4.** Immunoblot analysis of HSV-tagged SREBP-1a (B) and SREBP-2 (C) in 293 cells transfected with wild-type and linker region mutants. A, schematic diagram of the domain structures of SREBP-1a and SREBP-2, showing the sites of mutations in the linker region between amino acids 456/450 and residues 478–481 in SREBP-2) were each replaced by AS. For reference, the first amino acid in the first transmembrane domain is residue 488 and 482 for SREBP-1 and -2, respectively. B and C, immunoblot analysis of wild-type and linker region mutants of HSV-tagged SREBPs in nuclear extracts and membranes from 293 cells transfected with pTK-HSV-BP1a and its mutants (B) and pTK-HSV-BP2 and its mutants (C). On day 0, 293 cells were set up as described in the legend to Fig. 2. On day 2, the cells were transfected with 5 μg of the indicated plasmid. 3 h after transfection, the cells were switched to medium B in the absence (−) or the presence (+) of 1 μg/ml 25-hydroxycholesterol plus 10 μg/ml cholesterol (sterols) as indicated. On day 3, the cells were harvested and fractionated as described under “Experimental Procedures.” All aliquots of 40 μg of protein (B) or 60 μg (C) from nuclear extracts (upper panels) and aliquots of 60 (B) or 80 μg (C) from membranes (lower panels) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml HSV-Tag antibody. The filters in B and C were exposed to film for 30 s and 1 min, respectively. N and P denote the NH$_2$-terminal mature and precursor forms of SREBPs, respectively.

**Fig. 5.** Linker region mutations in SREBP-1a (A) and SREBP-2 (B) that do not affect cleavage or regulation. A, the bold letters denote amino acid residues that were individually changed to alanine and had no effect on cleavage or regulation in SREBP-1a. Asterisks denote point mutations that abolished or markedly decreased cleavage in SREBP-1a. B, the dashed line denotes residues in the linker region of SREBP-2 that were deleted and replaced by two amino acids (Ala-Ser for Δ433–477 and Δ402–469 or Thr-Gly for Δ433–449).

**Fig. 6.** Immunoblot analysis of HSV-tagged SREBP-1a (B) and SREBP-2 (C) from 293 cells transfected with wild-type and loop region mutants. A, schematic diagram of the loop regions of human SREBP-1a and SREBP-2, showing the sites of the mutated arginine (R) in both SREBPs. B and C, immunoblot analysis of wild-type and loop region mutants of HSV-tagged SREBPs in nuclear extracts and membranes from 293 cells transfected with pTK-HSV-BP1a and its mutants (B) and pTK-HSV-BP2 and its mutants (C). 293 cells were set up, transfected with the indicated plasmid, and fractionated as described in the legend to Fig. 2. All aliquots of 40 μg of protein (B) or 60 μg (C) from nuclear extracts (upper panels) and aliquots of 60 (B) and 80 μg (C) from membranes (lower panels) were subjected to SDS-PAGE and immunoblot analysis with 0.5 μg/ml HSV-Tag antibody. The filters in B and C were exposed to film for 30 s and 1 min, respectively. N and P denote the NH$_2$-terminal mature and precursor forms of SREBPs, respectively.
SREBP-1a (Fig. 7B). First, the amount of mature form in the nucleus was reduced. Second, there was no longer any suppression by sterols (Fig. 7B, compare lanes 5 and 6 with lanes 3 and 4). The sizes of the precursor form and the nuclear form were also reduced because the c class exons encode a shorter protein sequence than the a class exons. To determine whether the differences in cleavage and regulation were attributable to the sequence or the a class exons. To determine whether the resistance to sterol suppression is dictated by the c class exons at the NH2 terminus or COOH terminus, we prepared SREBP-1b, which contains exon 1a at the NH2 terminus and exons 18c and 19c at the COOH terminus (Fig. 7A). Cleavage of this version also failed to be suppressed by sterols, indicating that the resistance to sterol suppression is dictated by the exons at the COOH terminus (Fig. 7B, lanes 7 and 8).

DISCUSSION

In the current studies, we have developed an expression vector that produces physiologic amounts of SREBP-1 and -2, thereby allowing sterol-regulated cleavage to occur in transfected 293 cells. The amount of SREBP produced by this vector is about the same as the amount of endogenous SREBP in the cells (as judged from comparative immunoblots). Using this vector as a basis for a mutational analysis, we have identified two conserved regions of SREBP-1a and -2 that are required for high level cleavage by the sterol-regulated protease. Surprisingly, these two sequences lie on opposite sides of the ER membrane.

The most essential residue of either SREBP-1a or -2 is the Arg in the lumenal loop. Changing this Arg to an Ala abolished cleavage. In numerous experiments with this mutant protein, we have never observed any cleavage band, even when the gels were exposed for prolonged periods. Changing the Arg to a Lys in SREBP-2 preserved cleavage, indicating that the positive charge is the important feature. The precise location of this Arg is important because its removal abolished cleavage of SREBP-1a even though the lumenal loop of this protein contains two other arginines (Arg530 and Arg635). (The lumenal loop of SREBP-2 contains only one Arg.) We have not yet conducted an extensive mutational analysis of the lumenal loop to determine whether other residues are required. We note, however, that this sequence is not well conserved between SREBP-1a and -2 (Fig. 6A). One conspicuous conserved residue is a leucine that is three residues to the COOH-terminal side of the crucial Arg (Leu558 in SREBP-1a and Leu422 in SREBP-2). In experiments not shown, we changed this leucine to alanine in SREBP-2 without any effect on sterol-regulated cleavage.

The exact role played by the lumenal Arg is not clear as yet. It may be a site of initial cleavage that is followed by a second cleavage on the cytoplasmic side in the region of the DRSR sequence. It might also be part of a recognition sequence that binds a regulatory protein that facilitates cleavage on the cytoplasmic face. Several other possibilities exist, and they should be susceptible to analysis with the expression vector that is now in hand.

The requirement for the DRSR sequence on the cytoplasmic side of the membrane is not as absolute as the requirement for the lumenal Arg, at least for SREBP-2. The DRSR → AS mutant form of SREBP-1a never yielded a mature nuclear protein. However, in some experiments with the DRSR → AS version of SREBP-2, we observed a small amount of the nuclear form that was up to 10–20% of the amount generated by the wild-type protein in the same experiments. These data indicate that the DRSR sequence is important but not absolutely essential for sterol-regulated proteolysis. It may not be a cleavage site itself, but rather it may be part of the recognition site for a protease that cleaves elsewhere.

Aside from the DRSR sequence, the spacer region between the leucine zipper and the first transmembrane region does not appear to contain any residues that are crucial for cleavage of SREBP-1a or -2. As shown in Fig. 5B, we were able to delete all of the amino acids between residues 402 and 477 of SREBP-2 without affecting sterol-regulated cleavage. This eliminates all of the spacer region except for the DRSR sequence immediately adjacent to the membrane. A similar conclusion was drawn with regard to SREBP-1a. Here we observed normal sterol-regulated cleavage of mutant forms of SREBP-1a containing any of nine point mutations in the juxtamembrane portion (Fig. 5A). These substitutions included the GM sequence at positions 481 and 482, which is two residues to the NH2-terminal side of the DRSR sequence (Fig. 5A). Like the DRSR sequence, the GM sequence is conserved in human and hamster SREBP-1 and -2, but replacement of Gly or Met with Ala did not affect sterol-regulated cleavage in the transfected 293 cells.

The finding that cleavage of SREBP-1c is not regulated by sterols was a surprise, and it may have physiologic implications. Immunity to regulation was traced to the COOH-terminal two exons. We do not yet know whether the a class exons are required for regulation or whether the c class exons dominantly interfere with regulation. The COOH-terminal c class exons cannot be a major contributor to the total amount of SREBP-1 in tissue culture cells, because cleavage of endogenous SREBP-1 is totally suppressed by sterols (1, 13, 14). A different situation may pertain in liver. We previously observed that livers of chow-fed hamsters contain the nuclear form of SREBP-1 but not SREBP-2 (17). It is possible that this nuclear form results from the protein with the COOH-terminal c class exons. Inasmuch as we have no evidence for an alternatively spliced form of SREBP-2, cleavage of this protein may always be regulated by sterols. Experiments are currently underway to determine whether hamster liver contains mRNAs encoding the c class of SREBP-1.

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