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Calcium-binding protein, spermatid-specific 1 is expressed in human salivary glands and contains an anti-inflammatory motif

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St. Laurent CD, St. Laurent KE, Mathison RD, Befus AD. Calcium-binding protein, spermatid-specific 1 (CABS1) contains a similar sequence to that of the anti-inflammatory sequence in rat SMR1. Whether this similarity of CABS1 extends to the neuroendocrine regulation of the anti-inflammatory activity seen for SMR1 remains to be determined.

SMR1; Vcsa1; saliva; inflammation; neutrophilia

THERE ARE THREE MAJOR SALIVARY GLANDS in the oral cavity: the submandibular gland (SMG), sublingual, and the parotid. These glands have both exocrine and endocrine functions and produce and secrete numerous biologically active proteins, polypeptides, and hormones involved in growth and differentiation, homeostasis, and digestion. We have previously studied the prohormone submandibular rat 1 (SMR1), product of the Vcsa1 gene, which is highly expressed in the testes and salivary glands of rats, and can be cleaved to produce polypeptides with analgesic, erectile function, and anti-inflammatory activities. Humans lack the Vcsa1 gene, but homologous sequences and functions for analgesia and erectile function exist in the human genes Prol1, SMR3a, and SMR3b located on the human chromosomal region close to where Vcsa1 lies in the rat. Here we show the human protein calcium-binding protein spermatid-specific 1 (CABS1) contains a similar sequence to the anti-inflammatory sequence in rat SMR1, thus CABS1 may be another human gene with homologous function to Vcsa1. Using Western blot and PCR, we discovered that the human protein CABS1, previously thought to only be expressed in the testes, is also expressed in the salivary glands and lung, in a tissue-specific manner. Peptides derived from CABS1 were tested in an in vivo mouse model of lipopolysaccharide (LPS)-induced neutrophilia and an ex vivo rat model of antigen-induced intestinal anaphylaxis and significantly reduced both neutrophil accumulation in bronchoalveolar lavage fluid and antigen-induced ileal contractions, respectively. Thus human CABS1 has a peptide motif homologous to the anti-inflammatory peptide sequence of rat SMR1. Whether this similarity of CABS1 extends to the neuroendocrine regulation of the anti-inflammatory activity seen for SMR1 remains to be determined.

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lysates, and homogenization and processing were done at 4°C to reduce the likelihood of protein degradation. Protein lysates from human lung, spleen, and testes were purchased from Novus Biologicals (Littleton, CO), and CABS1 overexpression lysate and purified CABS1 protein were purchased from OriGene (Rockville, MD). RNA from human testes, SMG, and parotid was purchased from BioChain Institute (Newark, CA).

**RNA purification and PCR.** RNA was purified using the RNasequeous 4PCR kit, as per manufacturer’s directions (Life Technologies, Burlington, ON, Canada), and cDNA was made using the SuperScript III First-Strand Synthesis System using Oligo(dt)20 to prime the reaction (Life Technologies). Conventional PCR was done using 2 µg cDNA for salivary glands and 0.25 µg cDNA for testes, 2.5 µM of each primer (forward and reverse), 0.033 U/µl JumpStart REDTaq DNA Polymerase (Sigma-Aldrich), 200 µM dNTP mix, and 4.8 mM MgCl2 in each reaction. The annealing temperature was 60°C and 35 cycles were used. The products were loaded onto a 1.5% agarose gel containing ethidium bromide, run for 30 min at 100 V, and visualized under ultraviolet light. Primers used were for CABS1 were GCTGAAGATGGTTGCCCAAAT (forward) and CGGTCCCATATCATCCTGATTT (reverse), and for GAPDH, primers were CTGAGACGGAGCTGTTGCA (forward) and GCAATGAGCCCGAGCTTT (reverse).

**Gel electrophoresis and Western blot analysis.** Proteins were separated on 12% polyacrylamide gels (25 µg protein lysate was loaded for each tissue, and 1 µg for purified CABS1 protein) and transferred to 0.45/0.9262 M PB (pH 8.3) for 1 h at 100 V, and visualized under ultraviolet light. Primers used were for GAPDH, primers were CTGAGACGGAGCTGTTGCA (forward) and GCAATGAGCCCGAGCTTT (reverse).

**Protein identification by mass spectrometry.** Polyacrylamide gels of tissue extracts, overexpression lysates, or purified CABS1 were stained with Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories).

**Intestinal anaphylaxis animal model.** Rats were sensitized to ovalbumin (OA) with an intraperitoneal injection of 1 mg OA (Sigma-Aldrich) and 100 ng pertussis toxin (Sigma-Aldrich). Four to seven weeks later, rats were euthanized, the terminal ileum was excised, cut into eight 1.5-cm sections, and mounted in 20-ml organ baths containing bicarbonate Krebs buffer, under 0.75 g of tension. Tissues were allowed to equilibrate for 30 min and washed three times with buffer. Various concentrations of CABS1-derived and control peptides were added to the bath and incubated for 10 min, followed by antigen challenge by addition of 1 mg OA. The isometric force generated in response to OA was measured using a force displacement transducer (model FT03, Grass Technologies, West Warwick, RI). Tissues were then washed three times, baseline tension was reestablished, and peak contractile response was obtained by adding 10⁻⁵ M of the cholinergic agonist carbachol (Sigma-Aldrich). Data were recorded with Polyview software (Polybytes, Cedar Rapids, IA). The mucosa was then scraped from the tissue, and the mass of muscle was determined by wet weight. The OA response was first expressed as grams of tension per grams of muscle. This was then normalized to the peak contractile response to carbachol obtained for each ileal segment and expressed as the OA-to-carbachol ratio (22).

**LPS-induced lung inflammation animal model.** Mice were pre-treated with 100 µl of 5 mg/kg human or rat-derived peptides, or saline, orally by gavage. One hour later mice were lightly anesthetized with isoflurane (Benson Medical Industries, Markham, ON, Canada), and 60 µl of 500 µg/kg LPS from Escherichia coli serotype 055:B5 (Sigma-Aldrich) were given intranasally by droplets onto the nares. Twenty-four hours later, mice were euthanized, and bronchoalveolar lavage was performed by insertion of a tracheal catheter into the trachea. Lungs were washed 5× with 1 ml PBS, broncho-alveolar lavage fluid (BALF) was collected, and total cell counts were determined with a Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). White blood cells (WBC) differential counts were done by spinning 5,000 cells from the BALF onto a slide using a Shandon Cytospin 4 (Thermo Fisher Scientific). Slides were stained using the PROTOCOL Hema 3 staining system (Thermo Fisher Scientific). Three hundred cells from each slide were counted and used to determine the number of WBC in the original BALF sample. These experiments were conducted with two batches of mice that were
ordered 3 mo apart, as well as on several different days within each batch. To compensate for interexperimental variability of the LPS response, the results were normalized to our LPS-positive control group on each day.

Statistical analysis. One-way ANOVA with Dunnett’s multiple comparison test was used to assess statistical significance. Significance is represented as $P < 0.05$, $P < 0.01$, and $P < 0.001$. Statistical analysis and graphing were done using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA).

RESULTS

Identification of putative biologically active peptides derived from human CABS1. Using in silico analysis, we discovered that the human protein CABS1 contains the aa sequence TDIFELL near its COOH-terminus, which is very similar to the anti-inflammatory motif TDIFEGG located near the COOH-terminus of rat SMR1, a product of the Vcsa1 gene. In addition, CABS1 is located on human chromosome 4 adjacent to the genes PROL1, SMR3A, and SMR3B (Fig. 1, modified from Ref. 23), which have been found to contain similar aa motifs to that of rat sialorphin, another peptide derived from SMR1 with analgesic and erectile function activities. These genes are located in a region that is well conserved in mammals (26), containing gene products typically expressed in milk, testes, salivary and lacrimal glands, and enamel. Although there is no human homologue for the rat Vcsa1 gene, the four human genes PROL1, SMR3A, SMR3B, and CABS1 appear to have acquired two main functional motifs with biological activity found in Vcsa1.

Characterization of CABS1 expression in human submandibular glands. To determine whether CABS1 is expressed in human salivary glands, PCR and WB analyses were done on five human salivary gland samples obtained from the University of Alberta Hospital and commercially purchased SMG and parotid samples. All five human SMG samples and commercially purchased SMG RNA expressed CABS1 mRNA (Fig. 2). Purchased RNA from the parotid gland was also weakly positive for CABS1. Testes RNA was run as a positive control and showed high CABS1 expression, consistent with previously published studies in rats and mice (4, 14). Both male (Fig. 2, lanes 1, 3, 4, and 6) and female (Fig. 2, lanes 2 and 5) SMG were tested and showed similar expression patterns, indicating no obvious sexual dimorphism in human CABS1 RNA expression.

WB were done on the five SMG samples, and antibody H2 was used to detect CABS1 (Fig. 3A). The predicted relative molecular mass ($M_r$) of the translated polypeptide of CABS1 is 43 kDa. The major band detected in the SMG was at 51 kDa, 8 kDa higher than the predicted $M_r$. CABS1 is predicted to have several N- and O-glycosylation sites, but when treated with N- and O-glycanase, we did not observe a shift in the $M_r$ of any of the bands (data not shown). Other immunoreactive bands in the SMG were found at $M_r$ 33, 27 (doublet), 20, 17, 16, and 11 kDa; however, the bands at 17, 16, and 11 kDa were not present in all SMG samples (Fig. 3A). An additional band can sometimes be detected at 103 kDa, and its presence varies with the reducing agent used and the length of time the sample is boiled before running the WB. We detected no major differences in the expression pattern of CABS1 in SMG between males and females (Fig. 3A, lanes 1, 3, and 4 are male and lanes 2 and 5 are female), which is consistent with the mRNA data. Blocking controls for antibody H2 were done by incubating H2 with its immunizing peptide before applying it to the membrane, and no immunoreactive bands were detected using this control (data not shown).

CABS1 antibody specificity and expression in other tissues. WB results showed multiple immunoreactive bands in the SMG. To help validate these results, we examined four additional CABS1 antibodies (H1, H3, H4, and H5, detailed in the MATERIALS AND METHODS) using human SMG. Immunoreactive bands were consistently detected at $M_r$ 51, 33, and 27 kDa in all four additional antibodies (data not shown). One other weak band was detected at 59 kDa with three antibodies (H1, H3, and H4), but it was not present in all five SMG samples. Additional human tissues were also examined (Fig. 3A), as two previous studies have shown that CABS1 is expressed only in the testes and main olfactory epithelium (4, 14). Testes showed a similar banding pattern to SMG; however, the bands at 20, 17, and 16 kDa were not present. Interestingly, lung showed immunoreactive bands at only 33 and 27 kDa, but not at 51 kDa, which is predicted to be the full-length protein. There were no immunoreactive bands present in the spleen.

To confirm that the immunoreactive bands seen on WB are indeed CABS1, MS protein identification was used. The expression level of CABS1 in human SMG tissues is low, and we
were unable to detect it using MS analysis, therefore, a CABS1 overexpression lysate and purified CABS1 protein from this lysate were analyzed. With the use of WB analysis, the overexpression lysate showed a banding pattern for CABS1 consistent with SMG tissue, with the exception of an additional band at 75 kDa (Fig. 3B, lane 3). The purified protein contained the 75-kDa band and a weak band at 51 kDa (Fig. 3B, lane 4). There were no bands detected in the untransfected control lysate (Fig. 3B, lane 2). Analysis of the MS data confirmed the presence of CABS1 sequences in the immunoreactive bands in the overexpression lysate at 75, 51, 33, 27, 20, and 16 kDa, but not the 11 kDa band, and in all bands in the purified CABS1 protein. Sequence coverage for CABS1-positive bands was between 16 and 73%.

CABS1-derived peptides inhibit intestinal anaphylaxis. Three peptides derived from the human CABS1 protein (FELL, TDIFELL, and TDIFELLK) were tested in an animal model of antigen-induced intestinal anaphylaxis (Fig. 4). The peptides chosen have similar sequences to the biologically active rat SMR1-derived peptide that we have previously published, TDIFEGG, and the d-isomeric modified sequence feG (15–17). Consistent with previous studies, the negative control peptide fdG (Phe, Asp, Gly) did not reduce ileal contractions at either of the doses tested, and feG significantly reduced ileal contractions at both $10^{-6}$ M and $10^{-7}$ M (Fig. 4, A and B). The human peptide sequences FELL and TDIFELLK also significantly inhibited ileal contractions at $10^{-6}$ M and $10^{-7}$ M (Fig. 4, C and D). In contrast, TDIFELL did not

Fig. 3. CABS1 is expressed in human submandibular glands, testes, and lung. Protein was analyzed from five human SMG, testes, lung, and spleen (A) and SMG, control and overexpression (OE) lysates, and purified CABS1 (B). Lysate (25 μg) or purified CABS1 (1 μg) was run on 12% polyacrylamide gels. The membranes were blotted with CABS1 antibody H2. In A, male SMG samples are in lanes 1, 3, and 4 and female samples are in lanes 2 and 5. Numbers on left are the relative molecular mass (Mr) of each band in kilodaltons (kDa). Red bands are β-actin and green bands are CABS1.

Fig. 4. CABS1-derived peptides have anti-anaphylactic activity in an intestinal antigen challenge model. The terminal ileum was excised from OA-sensitized rats, and segments were mounted in an organ bath connected to a force displacement transducer. Ileal segments were incubated with two doses of peptides ($10^{-6}$ M and $10^{-7}$ M) for 10 min, followed by antigen challenge with 1 mg OA, and then ileal contractions were measured. Peak contractile response of each ileal segment was measured by adding $10^{-5}$ M carbachol, and all results are expressed as the OA-to-Carbachol contractile ratio. Peptides tested were fdG (Phe, Asp, Gly) (A), feG (Phe, Glu, Gly) (B), FELL (C), TDIFELLK (D), and TDIFELL (E); $n = 13–29$ animals in A and B and $n = 12–23$ animals in C–E. Significance is represented as *$P < 0.05$ and **$P < 0.01$. 

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significantly inhibit ileal contractions at either dose tested (Fig. 4E). Ileal contractions induced by carbachol alone (without antigen challenge) were not inhibited by any of the peptides (data not shown).

**CABS1-derived peptides inhibit lipopolysaccharide-induced neutrophilia in the lung.** In addition to the above peptides, (cyclohexylalanine)eG (AIM-102) was included as a control because it has previously been shown to be effective in this mouse model of LPS-induced neutrophilia in the lung (unpublished data; Aim Therapeutics). All three human peptides and AIM-102 significantly reduced total WBC accumulation in BALF (Fig. 5A). AIM-102 reduced WBC accumulation in the BALF by 39%, FELL reduced it by 51%, TDIFELL reduced it by 54%, and TDIFELLK reduced it by 57%. The negative control peptide fdG did not affect WBC accumulation in BALF. Of the total WBC population in the BALF, only neutrophil and macrophage numbers were affected. AIM-102 significantly reduced neutrophil numbers in BALF by 36%, feG by 53%, FELL by 52%, TDIFELL by 53%, and TDIFELLK by 56% (Fig. 5B). Macrophage numbers were significantly reduced in the TDIFELL (59%) and TDIFELLK (53%) groups, but not in the AIM-102 (27%) or FELL (29%) groups (Fig. 5C).

**DISCUSSION**

We have shown for the first time that CABS1 mRNA and protein is expressed in the SMG in both human males and females, and peptides derived from CABS1 have anti-inflammatory activity in models of intestinal anaphylaxis and LPS-induced neutrophilia. Previous reports have focused on CABS1 expression only in the testes of male mice and rats, where it functions as a calcium-binding protein involved in spermatogenesis (4, 14).

As we detected numerous immunoreactive bands in human SMG using WB (Fig. 3), we used several approaches to establish that the bands were CABS1 and not nonspecific. Five different antibodies developed to distinct CABS1 peptide sequences were used from three commercial suppliers, and the majority of the bands detected were consistent among the five antibodies, reducing the likelihood of those being nonspecific bands. These data were not shown because these additional antibodies had a low signal-to-noise ratio, which made cross-antibody comparisons of the low abundance bands difficult and potentially inaccurate. Blocking with immunogenic peptides and isotype controls were also done and showed no immunoreactive bands. Additional tissues were examined and immunoreactive bands were found in the testes, but not the spleen, which is consistent with previously published results in mice and rats (4, 14), and establishes tissue specificity in the detection of immunoreactive bands. Although Calvel et al. (4) and Kawashima et al. (14) showed only one band in their WB in the testes, our methodology for visualizing the bands using the Licor Odyssey Imager and infrared fluorophores is a more sensitive system for distinguishing low- and high-intensity bands on the same blot. The main band detected at 51 kDa was several orders of magnitude more intense than the weaker bands at 17, 16, and 11 kDa, and when the sensitivity levels on the Odyssey imager were reduced, only the 51-kDa band was detected.

We attempted to immunoprecipitate bands in SMG to confirm their identity by MS, but unfortunately we were unable to successfully immunoprecipitate enough material to get identification with MS. With the use of silver- or Commassie-stained 1- and 2-D gels, it is difficult to identify bands that are likely to be the immunoreactive bands we detected using WB, suggesting that the abundance of CABS1 protein is low. We then tested a transient overexpression lysate of CABS1, as well as purified CABS1 from this lysate, and found the banding pattern in the lysate to be almost identical to the SMG (Fig. 3B). The amount of CABS1 in this lysate was high enough for MS protein identification, and we found that all immunoreactive bands in the overexpression lysate except for the 11-kDa band contained CABS1. The 11-kDa band is in low abundance, and although it is immunoreactive with anti-CABS1 antibody,
CABS1 peptides were likely below the detection threshold for MS. We therefore believe that the immunoreactive bands we see in the SMG and other tissues are CABS1. Our results demonstrate that CABS1 is expressed in a greater range of tissues than previously reported and that the spectrum of immunoreactive bands may show some tissue specificity.

The predicted Mr of human CABS1 is 43 kDa, which is not a Mr of any of our immunoreactive bands. This is similar to SMR1 in the rat, which has a predicted Mr of 16 kDa, but immunoreactive bands can be detected between 16 and 25 kDa. It has been shown that SMR1 is N-glycosylated, and when treated with N-glycanase, the bands at 20–25 kDa shifted toward their predicted Mr of 16 kDa (24). We therefore postulated that the translated polypeptide of CABS1 could be N- or O-glycosylated, as it is predicted to have several glycosylation sites, but digestion of the protein with N- and O-glycanase did not result in a shift in the Mr of any of the detected bands, notably the 51-kDa band. The discrepancy in predicted versus observed Mr could be due to other posttranslational modifications on CABS1 or other innate properties affecting its mobility in a polyacrylamide matrix. Calvel et al. (4) found altered mobility of rat CABS1 during polyacrylamide electrophoresis (Mr, 79 kDa) due to the highly acidic nature of the protein, which was partially restored after treatment with carbodiimide and ethanolamine. Kawashima et al. (14) found mouse CABS1 also had altered mobility in a polyacrylamide matrix in testes (Mr, 66 kDa) and shifted downward to 58 kDa in cauda epididymal mature sperm, which was mediated by increased calcium binding (14). Interestingly, when our overexpression lysate was run through a column to obtain a purified CABS1 fraction, the major immunoreactive band present was 75 kDa. MS results on the 75- and 51-kDa bands showed excellent coverage of CABS1 in both bands (73% and 69%, respectively), and coverage extended in both cases to within eight aa of the COOH- and NH2-termini of CABS1, indicating that both bands likely contain full-length CABS1. This indicates that the disparity in Mr of the predicted mass of CABS1 and our (and others) experimental results for the immunoreactive bands at 75 and 51 kDa is most likely due to reasons outlined above. For the bands below 51 kDa, analysis of the CABS1 sequences detected by MS did not yield an obvious explanation for their differences; however, the sequence coverage was between 16% and 31%, and further work would have to be done to determine precisely which portions of CABS1 each band contained. Based on this evidence and the relative abundance of the 51-kDa band, it is likely that the 51-kDa band in SMG is the full-length polypeptide of human CABS1 in that tissue.

Like SMR1, CABS1 is also predicted to have many proteolytic cleavage sites, and its location in the salivary compartment exposes it to high levels of proteolytic enzymes, which could result in CABS1 being cleaved into smaller protein fragments. The numerous bands detected are reminiscent of SMR1 in rat SMG, which is cleaved into many smaller protein fragments (24), some of which have distinct biological activity (28), and can be released into the saliva and bloodstream (29, 30). We have preliminary evidence that CABS1 is present in human saliva and plasma (data not shown). We do not, however, currently know if the anti-inflammatory peptide sequences FELL, TDIFFELL, and TDIFFELLLK are present as biologically active individual fragments, only that we can detect immunoactive polypeptides greater than 11 kDa that contain them.

As CABS1 has previously been reported to be expressed only in the testes (4, 14), we investigated both male and female SMG tissue to see if its expression was sexually dimorphic. Expression of both RNA and protein was similar between male and female samples, and hence we detected no sexual dimorphism of CABS1 in the SMG. This is in contrast to rat SMR1, which is highly expressed in the male sex organs and has 1,000-fold higher expression in male SMG compared with female glands (27). As CABS1 was first discovered as a calcium-binding protein involved in spermatogenesis in males, its expression in both male and female salivary glands may indicate it has other functions in vivo, which may include the anti-inflammatory action of peptides derived from CABS1.

Although we found that all three CABS1-derived peptides tested have anti-inflammatory activity (Figs. 4 and 5), the results between the two animal models differed. In the LPS-induced lung injury mouse model, all human peptide sequences tested significantly reduced both total cell and neutrophil numbers in BALF. We postulate that the aa sequence FELL contains the core anti-inflammatory component, which is similar to TDIFFEGG from rat SMR1, in that the aa sequence FEG is the minimum sequence required for its anti-inflammatory activity (15). In the intestinal anaphylaxis rat model, however, the human peptide TDIFFELL failed to significantly reduce ileal contractions at any of the doses tested, while FELL and TDIFFELLLK both acted in at least two of the doses. Unfortunately we do not currently know the mechanism of action of our peptides, so we are unable to determine whether the differences between the two assays are due to species variation, differential binding affinities of the peptides to their targets in these models, or lower sensitivity and greater variability of the intestinal anaphylaxis model. There is evidence that neutrophils are involved either directly or downstream from the action of the anti-inflammatory peptides from rat SMR1 (2, 11, 18, 20), and this could help explain why the human peptides have greater efficacy in the LPS-induced neutrophilia model in the mouse than in an anaphylactic assay, which has, at least until recently (13), been thought to have little or no neutrophil involvement. We have also observed differences with various peptide sequences from rat SMR1 in their activities in distinct inflammatory models (19, 22, 31). We therefore postulate that FELL and the other human CABS1-derived peptides will have activity in a range of anti-inflammatory assays and disease models.

**Perspectives and Significance**

We have shown that CABS1 contains an anti-inflammatory motif that is able to reduce inflammation and anaphylaxis in two animal models. This motif is likely a functional homologue of the anti-inflammatory motif found in rat SMR1 and thought to be under the control of neuroendocrine pathways. CABS1 is expressed in humans in several tissues in both sexes, notably the SMG, where it may be processed in the salivary compartment to liberate peptide fragments with anti-inflammatory activity that could be the basis for an endogenous human anti-inflammatory pathway with both endocrine and exocrine functions. Further study of CABS1 in human saliva and blood.
to characterize its biologically active peptides and to study their regulation and function would be of value.

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DISCLOSURES

A. D. Befus and R. D. Mathison have a very small equity position in, and have previously received consulting fees from AIM Therapeutics, a start-up biotech company with patents on anti-inflammatory peptides derived from the rat pro-hormone SMR1.

AUTHOR CONTRIBUTIONS

Author contributions: C.D.S., K.E.S., and A.D.B. conception and design of research; C.D.S. and R.D.M. performed experiments; C.D.S., K.E.S., and R.D.M. analyzed data; C.D.S., K.E.S., and R.D.M. interpreted results of experiments; C.D.S. prepared figures; C.D.S. drafted manuscript; C.D.S., K.E.S., R.D.M., and A.D.B. edited and revised manuscript; C.D.S., K.E.S., R.D.M., and A.D.B. approved final version of manuscript.

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