Temporal Expression Pattern of Fkbp8 in Rodent Cochlea

Magdalena Zak¹, Andreas Bress², Markus Pfister² and Nikolaus Blin^{1,3}

¹University of Tübingen, Institute of Human Genetics, Tübingen, ²University of Tübingen, Department of Otorhinolaryngology, THRC, Molecular Genetics, Tübingen, ³Medical University Wroclaw, Dept. of Genetics, Wroclaw

Key Words

Gene activity • Cochlear maturation • Mouse • Rat • Tectorial membrane • Stria vascularis

Abstract

Background: FKBP8 is a multifunctional protein involved in many distinct processes like formation of central nervous system, viral RNA replication and inhibition of apoptosis. Fkbp8 expression was reported in different tissues, various cell lines and malignancies, in the latter displaying changes during carcinogenesis. Loss of Fkbp8 leads to substantial neurodegenerations during regular mouse development, thus hearing onset in mice could also potentially depend on *Fkbp8* expression. Since Fkbp8 is crucial for patterning of neuronal function, we studied its expression during maturation of the rodent auditory function. **Methods:** *Fkbp8* gene expression in rodent cochlear samples was studied by RT-PCR, qPCR, and western blot. Localization of *Fkbp8* transcripts and protein was analyzed by *in-situ* hybridization and immunohistochemistry. **Results:** Studies of auditory organ demonstrate that *Fkbp8* gene activity is increasing just before hearing onset and gradually decreasing after onset of hearing.

Western blot analysis suggests substantial levels of Fkbp8 protein before hearing onset, and slow degradation after onset of hearing. The *Fkbp8* mRNA is localized in spiral ganglion of cochlea but its distribution changes over time to the stria vascularis, a finding supported by immunohistochemistry staining. Additionally, in pre-hearing time Fkbp8-specific signal was also observed in the tectorial membrane, whose α - and β -Tectorin components show similar time-dependent expression of mRNA as *Fkbp8*. **Conclusion:** These results indicate a temporal shift in expression of Fkbp8 which correlates with cochlear maturation, strongly suggesting a contribution of Fkbp8 to the onset of the rodent hearing processes.

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Introduction

FK-506-binding protein 38-kDa (Fkbp8, also known as Fkbp38) is a member of the FKBP family of immunophilins. Even though its amino acid sequence shows high similarity to the other immunophilins, it is unique among other FKBP family members [1]. In contrast to the other members of the family, Fkbp8 does not display typical

properties of immunophilins like peptidyl prolyl *cis-trans* isomerase (PPIase, EC 5.2.1.8) activity and affinity for immunosuppressive drugs such as cyclosporine A (CsA), rapamycin, and tacrolimus (FK-506). The amino-terminal region of Fkbp8 contains a FKBP-like domain, a conserved motive among FKBP, responsible for their enzymatic activity as well as for binding to FK506. In Fkbp8 this domain has several amino acid substitutions at a few important positions leading to a change of its biochemical properties [1].

Like other immunophilins, Fkbp8 contains several additional domains: a tetratricopeptide repeats (TPRs) domain in the C-terminal region that mediates protein-protein interaction, the FKBP-C domain, a Ca²⁺/calmodulin binding domain, and a transmembrane domain localized at the C-terminus [1, 2]. The potential functions of Fkbp8 are influenced by the number of structural domains and increasing data suggest Fkbp8 to be a multifunctional protein, involved in the regulation of many different cellular processes.

Fkbp8 targets and anchors anti-apoptotic molecules Bcl-2 and Bcl-x_l to the mitochondria and inhibits calcineurin activity in FK506 immunosuppressant-free manner, whereas other FKBP are able to inhibit only in presence of FK506 [3]. Okamoto et al. showed that Fkbp8 interacts with Hepatitis C virus (HCV) non-structural protein 5A (NS5A) and recruits heat shock protein 90 (HSP90) to form a complex participating in viral RNA replication [4]. New studies demonstrate that NS5A through binding to Fkbp8 becomes additionally involved in programmed cell death inhibition [5]. Fkbp8 plays a role in size regulation of mammalian's cell, binds to and acts as an endogenous inhibitor of mTOR, and is required to antagonize Sonic Hedgehog signaling in forming of central nervous system [6-8].

Studies using *Fkbp8*^{-/-} mutant mice have shown importance of Fkbp8 for neuronal tissues. Eye neurodegenerations and malformations in neural tube development during embryogenesis observed by Bulgakov et al. [8] encouraged us to investigate the significance of *Fkbp8* for the auditory system. The most critical structure in the auditory pathway is the cochlea – a part of the inner ear involved in transformation of mechanical waves created by sound to electric signals in neurons. Not all animals are able to hear from the very first days of their lives, rodents are born deaf and develop hearing during cochlear maturation. This development is strongly connected with changes of expression patterns of specific components and *Fkbp8* also demonstrates such hearing-stage related activity.

Materials and Methods

Animals

Male and female wistar rats obtained from Jackson Laboratories (Bar Harbor, Maine, USA) and previously described wild-type *Tff3*^{+/+} male and female mice were used for this study [9].

Before decapitation, animals were asphyxiated with ether. Care and use of the animals as well as the experimental protocol were reviewed and approved by the animal welfare commissioner and the regional board for scientific animal experiments in Tübingen.

Isolation of murine RNA and RT-PCR

After decapitation cochleas were isolated and dissected as previously described [10]. For RNA and protein isolation, tissues were dissected, immediately frozen in liquid nitrogen and stored at -80°C before use. The RNeasy Kit (Qiagen) was used to isolate total RNA from various mouse tissues including cochleas of 6-14 mice pooled together in each sample. After RNA isolation reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, München, Germany), according to the manufacturer's instruction. The cochlear PCR were performed using *Fkbp8**co* forward 5'-AGC TGC TAC AGC TGA AGG TCA AGT-3' and reverse 5'-GCA GCA CGC TTC TTT ACC AGC TTT-3' primers, *Cyclophilin A* primers were used as an internal control.

Real-time quantitative PCR

A 105 bp fragment of mouse *Fkbp8* was amplified by real-time PCR with the oligonucleotides: forward primer 5'-AAC ATC AAG GCA CTG TTC CGC A-3' and reverse primer 5'-TGT TGG AAG GTT CCA GCT TCA GG-3'. For normalization following primers were used: *Cyclophilin A* forward 5'-CGA GCT CTG AGC ACT GGA GAG AAA-3' and reverse 5'-CAT GCC TTC TTT CAC CTT CCC AAA GAC-3', *mGapdh 4* forward 5'-AAC GAC CCC TTC ATT GAC-3' and reverse 5'-TCC ACG ACA TAC TCA GCA C-3' primers [11]. Each PCR mix contained: 100 ng of cDNA, 10 nM concentrated primers and 12,5 µl of 2x QuantiFast SYBR Green PCR Master Mix (Qiagen, Germany) in a 25 µl reaction volume. PCR was performed according to the Qiagen real-time cycler condition, using the iCycler iQ machine (Bio-Rad, USA). Before each run 1x External Well Factor Solution (Bio-Rad, USA) was used. The results were analyzed using Bio-Rad iQ5 software. All tissues and time points in cochlear maturation were measured in triplicates. The analyses were performed four times. The normalization and relative quantification using 2^{-ΔΔC} method were calculated after Livak and Schmittgen, as well as Liu and Saint [12, 13].

Statistical analysis

For statistical analyses the software SPSS 19.0 was used. For tests on normality the Kolmogorov-Smirnoff test was used. Due to lack of normality and homogeneity of variance the Kruskal-Wallis H-test was performed. Differences associated with p values < 0,05 were considered to be statistically significant. All data are presented as means ± standard deviation.

Fig. 1. Activity of *Fkbp8* in rodent cochlea. By RT-PCR *Fkbp8* was detected only in cochlea P10. cDNA omitted as negative control (N).

tion. Asterisks used to show significance in graphs (* $\leq$ 0,05; ** $\leq$ 0,01; *** $\leq$ 0,001).

Western blot

RNAlater stabilized cochlea samples (8-14 cochleas pooled together in one sample) were homogenized and lysed in lysis buffer (50 mM Tris-HCl pH 7,6, 150 mM NaCl, 2 mM EDTA, 0,5% Triton X-100) containing Pefabloc (10 μ l/ 5 ml, Sigma-Aldrich). Nuclei and cell debris were pelleted by centrifugation at 300x g for 5 min at 4°C and the supernatant was used for western blotting.

The denatured protein samples were separated under the reducing conditions by electrophoresis in 12% SDS-polyacrylamide gel, as a protein marker All Blue Standard from Bio-Rad (München, Germany) was used. After separation, proteins were transferred onto 0,2 μ m PVDF transfer membranes. Afterwards, membranes were blocked using 1x Blocking Buffer (Sigma-Aldrich) for 1 hour at room temperature, and incubated with the primary antibody at 4°C overnight. We used the following antibodies: an affinity-purified section 4 polyclonal antibody from rabbit against the purified FKBP8 domain (amino acids 1–165) (kind gift of Ph.D. Frank Edlich, Surgical Neurology Branch, NINDS, National Institutes of Health, Porter Neuroscience Research Center, 1:5000) and polyclonal anti-Actin A (20-33) antibody from rabbit (Sigma-Aldrich, 1:2500). On the next day, membranes were incubated with the secondary antibody for 1 hour at room temperature. Anti-Fkbp8 in brain was detected by AP Conjugate, Anti-(Rabbit IgG) raised in Goat (1:10000, Applied Biosystem, Carlsbad, California, USA) and visualized using CDP Star Chemiluminescent Substrate for Alkaline Phosphatase (Applied Biosystem, Carlsbad, California, USA). GE Healthcare ECL anti-rabbit from donkey IgG, Horseradish Peroxidase linked, GE Healthcare (1:2000) secondary antibody was used in cochlear studies. The results were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection Reagent (GE Healthcare, Uppsala, Sweden).

Tissue preparation

Cochlear sections were prepared as previously described by Schug et al. [14] and stored at -80°C. Briefly, tissues were fixed by immersion in 2% paraformaldehyde, 125 mM sucrose in 100 mM phosphate buffered saline, pH=7,4 (all chemicals from Sigma-Aldrich, Germany), for 2 hours and incubated overnight in 25% sucrose and 1 mM protease inhibitor (Pefabloc; Roche, Germany) in phosphate buffered saline, pH=7,4. After fixation, cochleas obtained from animals older than P10 were decalcified for 15 min to 2 hours with RBD buffer. In the next step, the tissues were embedded in O.C.T. compound (Miles Laboratories, USA), cryosectioned at 10 μ m, mounted on SuperFrost* / plus microscope slides, then

dried for 1 hour, and stored at -20°C before use.

In situ hybridization

The mouse *Fkbp8*-specific riboprobes were obtained using forward 5'-CAC CTC CAA CAC CAA AGT GGA CAT-3' and reverse primers 5'-GAC AGT CAG TTC CTG CCA GCA AT-3' (annealing temperature: 58°C, 35 cycles). The 491 bp long PCR fragment was cloned into the pCR-II-Topo Dual Promotor Vector (Invitrogen, USA) and used for *in vitro* transcription. Complementary strands for sense and antisense were transcribed from either SP6 or T7 promoter sites in the presence of digoxigenin-labelling mix (DIG; Roche, Germany).

Fixed sections were thawed and riboprobes (1:200) were applied for 5 hours hybridization at 58°C. On the next day the sections were washed three times in 0,1% SSC buffer (3M NaCl, 0,3M Tris-HCl-citrate) at 58°C for 30 minutes, blocked with the blocking solution (10% Blocking reagent (Boehringer, Ingelheim am Rhein, Germany) diluted in Malic acid (Sigma-Aldrich) and stained by the DIG antibody coupled to alkaline phosphatase (Roche, 1:750) for 30 min at 37°C. Afterwards slides were incubated with the visualization substrate composed of BCIP (Sigma-Aldrich), NBT (Roche) and AP-buffer (100 mM Tris-HCl, pH=9,5, 100 mM NaCl). The results of *in situ* hybridization were analyzed on Zeiss Axioplan 2 imaging microscope using 40x (N.A. 0.75), 20x (N.A. 0.50) objectives and 25x (N.A. 0.80) oil-immersion lens.

Immunohistochemical staining

The frozen tissue sections were thawed and dried at room temperature for 30 minutes followed by permeabilization with 0,1% Triton X-100 in PBS for 3 min. In the next step, cochleas were blocked with 2% BSA in PBS for 1 hour at room temperature and incubated overnight at 4°C with primary antibodies diluted in 0,5% BSA in PBS. The rabbit polyclonal anti-Fkbp8 (Abcam, ab24450: 1:50) antibody was used. On the next day, primary antibody was detected with Cy3- (Jackson Immuno Research Laboratories, USA 1:1500) conjugated secondary antibody by incubation for 1 hour at room temperature. Slides were rinsed 3 times in PBS, and mounted in Vectashield containing the nuclear stain DAPI (Vector Laboratories, USA). The results of immunohistochemistry were analyzed using Zeiss Axioplan 2 imaging microscope equipped with epifluorescence.

Results

Expression of *Fkbp8* in cochlea

Based on reported presence in various organs, e.g. kidney, brain, lung, heart and ovary, *Fkbp8* is considered to be a ubiquitously expressed protein [8]. Here, we

Fig. 2. Quantification of *Fkbp8* expression. A) The regulated expression of *Fkbp8* in cochlea reaches the highest level at P10 sample and is down-regulated after-onset of hearing. To each maturation time point the mean of (n=12) $2^{-\Delta\Delta C}$ values were taken ($\pm$ 1SE, p value). Expression is given with respect to the transcription level in cochlea P10. B) At postnatal day 10 *Fkbp8* is highly expressed in cochlea and heart, whereas in kidney, brain and skeletal muscle the levels are almost not traceable. For different tissues the mean of (n=12) $2^{-\Delta\Delta C}$ values were taken ($\pm$ 1SE, p value). Expression is given with respect to the transcription level in brain P10.

studied *Fkbp8* transcripts in rodent cochlea. Rodents, unlike humans, are born deaf and develop their hearing with time. Thus, we prepared cochlea samples representing time points before hearing onset (postnatal day (P) 5, P10) and after hearing onset (P16, P23, P33, P41). RT-PCR indicated that *Fkbp8* expression is regulated during maturation of the auditory system. The PCR product was detected only in P10 sample (Fig. 1).

The analysis of *Fkbp8* expression was extended by quantitative PCR. The same set of pre-hearing (P5, P10) and post-hearing (P16, P23, P33, P41) cochlea samples demonstrated presence of *Fkbp8* in P5, P10, P16 and

A	maturation time point	test statistic	df	p value
cochlea P10	cochlea P5	-31.917	4	< 0.001
cochlea P10	cochlea P16	12.000	4	< 0.050
cochlea P10	cochlea P23	28.083	4	< 0.001
cochlea P16	cochlea P5	-19.917	4	< 0.001
cochlea P16	cochlea P23	16.083	4	< 0.010

B	tissue	test statistic	df	p value
brain P10	cochlea P10	31.182	4	< 0.010
brain P10	heart P10	32.182	4	< 0.010
skeletal muscle P10	cochlea P10	38.500	4	< 0.001
skeletal muscle P10	heart P10	39.500	4	< 0.001
skeletal muscle P10	kidney P10	21.000	4	< 0.030

Table 1. p values by Kruskal-Wallis H test. A compared pairwise of the mean of $2^{-\Delta\Delta C}$ values at different maturation time points of cochlea (A) or different tissues (B) in rodent using H test (df = degrees of freedom).

Fig. 3. Expression of *Fkbp8* protein in cochlea. Using a polyclonal antibody against *Fkbp8* both protein isoforms were recognized in mouse brain and cochlea. After onset of hearing *Fkbp8* signals in cochlea became gradually weaker. Anti-actin antibody was used as an internal control.

P23 samples. The highest expression level was observed in cochlea P10. *Fkbp8* signal was gradually decreasing in P16 and P23 and became very low, if any, in P33 and P41 (Fig. 2A). Statistical significance is shown using the Kruskal-Wallis H test (Table 1A). This pattern of transcriptional activity suggests that *Fkbp8* may be involved in the maturation of the rodent auditory system.

We compared the expression intensity in cochlea P10 with other mouse tissues at this time point. Quantitative PCR results calculated with respect to the transcription level in brain showed that, next to a significant activity of

Fig. 4. Localization of *Fkbp8* transcripts in rodent cochlea. *Fkbp8* transcripts are localized in cochlear spiral ganglion and with time broaden their presence to stria vascularis. Scale bar: 20 μ m.

Fig. 5. Distribution of *Fkbp8* protein within murine cochlea. Immunohistochemistry staining revealed, next to spiral ganglion (white arrow), strong *Fkbp8* staining (yellow) in tectorial membrane of mouse P6. In post-hearing cochlea, *Fkbp8* was seen in stria vascularis (white arrow), whereas spiral ganglion and tectorial membrane remained unstained. Cell nuclei were counterstained with DAPI. Scale bar: 20 μ m.

Fkbp8 in cochlea and heart, kidney also showed a substantial expression. In skeletal muscle however the

activity was almost absent (Fig. 2B). The only organ in which we did not observe a signal was liver (data not

shown). Statistical significance is shown using the Kruskal-Wallis H test (Table 1B).

An antibody against Fkbp8 was used to confirm presence of both protein isoforms in brain and cochlea. When tested in mouse brain, we observed two signals at an apparent molecular weight of 52 kDa for the shorter isoform and 56 kDa for the longer one (Fig. 3), whereas the calculated molecular weights of both forms of murine Fkbp8 are 44 kDa and 46 kDa [15]. This suggests a post-translational modification, a fact also reported by Bulgakov et al. [8]. In samples representing pre- (P4, P10) and post-hearing onset (P15, P19) the cochlear protein pattern was comparable to the brain (Fig. 3). The protein level also reflects the expression changes of Fkbp8 in cochlea. On this level we did not observe any noticeable changes between P4 and P10 samples, but the Fkbp8 signals became gradually weaker in cochlea P15 and P19. This implies that Fkbp8 is significantly synthesized in murine cochlea shortly after birth, and during the onset of hearing, and the protein is slowly degraded afterwards.

Localization of Fkbp8 transcripts in cochlea

To specify the activity of *Fkbp8* in different cell types of the inner ear, the cochlear localization of the mRNA was assessed by *in-situ* hybridization with specific riboprobes. A strong, intracellular staining was seen in spiral ganglion of rat P5 (Fig. 4) whereas signals in the organ of Corti, basilar membrane and in the other structural elements of cochlea were very weak, if any. Animals 3 days older showed that *Fkbp8* broadens its distribution with time. In rat P8 we detected *Fkbp8* mRNA in the spiral ganglion and in stria vascularis of the cochlear duct (Fig. 4). However, the observed intensity of *Fkbp8* transcripts in spiral ganglion was weaker than in P5 cochlea. Expansion of *Fkbp8* in cochlea over this particular time span correlates well with the quantitative PCR data indicating an increase from postnatal days 5 to 10.

Distribution of Fkbp8 protein within murine cochlea

The localization study in cochlea was extended to immunohistochemistry staining. Surprisingly, before onset of hearing (P6), next to spiral ganglion, very strong Fkbp8-specific signals were observed in tectorial membrane, whereas stria vascularis, basilar membrane, organ of Corti and other structural elements remained unstained (Fig. 5). During cochlear maturation, expression of Fkbp8 changes in accordance with the trends previously reported by *in-situ* hybridization and Western blot. While Fkbp8 was observed in stria vascularis, very weak, if any signals

were detected in spiral ganglion of mouse P18 (Fig. 5). After onset of hearing the tectorial membrane remained unstained.

Discussion

Fkbp8 is a ubiquitously expressed, multifunctional protein involved in distinct cellular processes [3, 4, 6-8]. The gene coding for Fkbp8 maps to a locus which has not been associated with any type of hearing loss, but this protein has a significant meaning for neurosensory systems. The importance of Fkbp8 for neuronal tissues was shown in knock-out mice which displayed malformations in eye and neural tube development during the embryonic stages [8]. Interestingly, these animals did not show any malformation in the non-neuronal tissues and the phenotypic features observed in *Fkbp8*^{-/-} mice could not be assigned to increased apoptosis.

For the first time we demonstrated Fkbp8 in auditory organ. According to our results, *Fkbp8* is present in cochlea and shows an expression pattern dependent on maturation of hearing. Additionally, over time Fkbp8 protein demonstrates alteration in its distribution within cochlea. The presence of Fkbp8 initially observed in spiral ganglion and tectorial membrane changes to stria vascularis, which points to different function of the protein in this auditory organ.

For several years it has been known that cochlear tectorial membrane is a ribbon-like stripe of extracellular matrix made of several genetically different types of collagen; type II, V, IX, XI and three non-collagenous proteins; α -Tectorin (*Tecta*), β -Tectorin (*Tectb*) and Otogelin (*Otog*) [16-19]. Our studies revealing Fkbp8 as a fourth protein component of the tectorial membrane, shed a new light on the complexity of its structure. The Fkbp8-specific staining observed in this extracellular matrix during the time of membrane formation suggests a contribution of the protein to this process. Interestingly, the genes coding for two major non-collagenous proteins α - and β -Tectorin demonstrate expression patterns similar to *Fkbp8*. They are highly active during the early stages of cochlear maturation. Afterwards, their mRNA level decreases in post-hearing time becoming undetectable at postnatal day 22 [20, 21]. However, in contrast to Fkbp8 both Tectorins remain structurally present in the tectorial membrane after onset of hearing [22].

Since mutant mice showed developmental malformations in eye and central tube it is very tempting to speculate whether Fkbp8 impairment can influence the

maturation of hearing. The tectorial membrane in response to sound-induced vibration of the basilar membrane causes deflection of stereocilia and propagates longitudinal travel of waves at audio frequencies [23]. The data obtained from *Tecta*^{-/-} and *Tectb*^{-/-} mice associate the abnormal morphology of the membrane and impairment of outer hair cells function with the moderate to severe hearing loss, whereas *Otog*^{-/-} mouse model demonstrates varying degrees of deafness and very strong balance defects [24-27].

The imbalanced movement of Otogelin deficient mice underlies connection between auditory and vestibular organ in mouse. The macula contains another extracellular matrix, called otoconial membrane, which is composed of the same three non-collagenous proteins [22]. Therefore, expanding research to the vestibular organ would help to elucidate the importance of Fkbp8 for the body balance.

Not surprisingly, before onset of hearing Fkbp8 is localized in spiral ganglion of cochlea – a group of nerves responsible for sending converted sound signals to the brain. Over time Fkbp8 changes its distribution to stria vascularis which is a source of potassium-rich endolymph. We hypothesize that the function of Fkbp8 in stria vascularis is related to potassium channels. Walker et al. [28] proposed a co-chaperone role of Fkbp8 together with Hsc70/Hsp90 system for the trafficking of hERG potassium channels in heart. Fkbp8 not only interacts with the hERG but also influences its trafficking efficiency.

The main driving force for sensory transduction in cochlea depends on potassium cycling. The regulation of K⁺ concentration in stria vascularis is crucial for formation of endocochlear potential, a positive voltage of 80-100 mV that is essential for audition [29]. Thus, the deficiencies in potassium channels localized in stria vascularis abolish the endocochlear potential and cause hearing impairment [30-35]. Therefore, a cochlear expression peak for *Fkbp8* at postnatal day 10 and presence in tectorial membrane just before hearing onset

together with the localization of Fkbp8 in the stria vascularis support the idea of the protein's contribution to the maturation of the hearing.

Presence of Fkbp8 in spiral ganglion neurons, despite being short-termed, becomes even more interesting in the light of the facts that FKBP proteins may mediate neuroprotective and neuroregenerative effects [36]. Influence of Fkbp8 inhibition on the regulation of neuronal cell death and proliferation can be a starting point for investigation of Fkbp8 related neuroprotection of auditory nerves [37].

Surprisingly, at day P10, cochlea is one of the organs with the strongest *Fkbp8* expression. Only heart had a higher level of *Fkbp8*, whereas in kidney, brain and skeletal muscle signals were at lower level. Presence of Fkbp8 in these non-sensory tissues was already presented by Bulgakov et al. who however used immunoblotting without quantification via qPCR [8]. Their western blots using lysates from adult animals of unknown age indicate that the heart generates a much weaker signal than brain or kidney, whereas liver is one of those tissues where the strongest immunoreaction of Fkbp8 antibody was reported.

The significance of Fkbp8 function in cochlea requires further elucidation, thus the genetically impaired *Fkbp8* mouse mutant model, showing different neurodegenerative problems, should allow more detailed studies of the protein contribution to the hearing process [38].

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