CFTR in K562 human leukemic cells

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Am J Physiol Cell Physiol 285: C480–C488, 2003; 10.1152/ajpcell.00320.2002.—In this study, the expression and functional characterization of CFTR (cystic fibrosis transmembrane regulator) was determined in K562 chronic human leukemia cells. Expression of the CFTR gene product was determined by RT-PCR and confirmed by immunohistochemistry and Western blot analysis. Functional characterization of CFTR Cl− channel activity was conducted with patch-clamp techniques. Forskolin, an adenylyl cyclase activator, induced an anion-selective channel with a linear current-voltage relationship and a single-channel conductance of 11 pS. This cAMP-activated channel had a P[^glucose]/P[^Cl] or P[^F]/P[^Cl] perm-selectivity ratio of 0.35 and 0.30, respectively, and was inhibited by the CFTR blocker glibenclamide and the anti-CFTR antibody MAb 13-1, when added to the cytoplasmatic side of the patch. Glibenclamide decreased the open probability increasing the frequency of open-to-closed transitions. Addition of 200 μM DIDS caused an irreversible block of the channels when added to the cytosolic side of inside-out patches. These and other observations indicate a widespread distribution of CFTR gene expression and suggest that this channel protein may function in most human cells to help maintain cellular homeostasis.

CFTR; K562 cells; leukemia cells; ion channels; glibenclamide; DIDS; PCR; immunohistochemistry

CHRONIC MYELOID LEUKEMIA is a malignant chronic disorder of hematopoietic stem cells that results in increased myeloid, erythroid, and platelet cell count in peripheral blood. The natural history of this disease is a progression from a benign chronic phase to a rapidly fatal blast crisis. The human myeloid cell line K562 was established by culturing leukemic cells from the pleural effusion of a patient in blast transformation. K562 cells are arrested in very early stages of development and provide a unique population of primitive human leukemia cells that can be induced to differentiate along with erythroid, monocye-macrophage, and megakaryocytic lineages in response to various stimuli (20, 28).

Previous studies in K562 cells reported the activity of Cl− (50), Ca, and Na channels (29), mechanosensitive cation channels (43), and a Ca-dependent Na channel regulated by the actin cytoskeleton (32). K562 cells also express cystic fibrosis transmembrane regulator (CFTR) mRNA transcripts (51) and P-glycoprotein (50). According to this, we recently reported (3) in K562 cells the presence of an anionic channel with an outward rectification (ORCC), a channel regulated by the CFTR (21, 37).

Although it is not known at what stage of development these channels first appear in blood cells, ion channel activity is involved in the mechanism of action of antileukemic agents that induce differentiation in K562 cells (20). Aberrant expression of ion channels has been detected in tumors of nonhematopoietic and hematopoietic origins (41). In other blood cell types, ion channels are related to T cell activation, activation of leukocytes by inflammatory stimuli, and cell volume regulation (10, 25). These findings stress the importance for their role in cell transformation and therapeutic potential value (41).

The CFTR, a member of the ABC superfamily of transporters, is a cAMP-activated chloride channel (34, 37). It was proposed that CFTR conducts or regulates ATP movement (see also Ref. 7), maintaining cellular anion secretory properties (14). CFTR also regulates other ion channels, including ORCC and epithelial Na channels (37; reviewed in Ref. 21). CFTR enhances apoptosis due to regulation of the Cl−/HCO3− exchanger (4). Mutations in the CFTR gene cause cystic fibrosis, a genetic disease characterized by defective functions of Cl− and other types of channels (2).

By providing abundant amounts of cells, the K562 line can serve as a useful model for cells representing the different stages of maturation and also for testing agents that induce terminal differentiation (28) or as target for the development of antileukemic agents (20). Our long-term goals include defining channels as markers for various stages of cell differentiation, evaluating the regulatory mechanisms of channel expression, and exploring possible functional correlates of different patterns of ion channel expression. Taking into account all these considerations, we found it of interest to determine whether in K562 cells, which may become resistant to cytostatic drugs and are capable of differentiation, ABC-type proteins can be expressed and whether they have a role as ion channels. The patch-clamping studies and the RT-PCR and Western blot experiments demonstrated the presence in K562 cells of a cAMP-stimulated anion channel with...
characteristics consistent with the presence of a functional CFTR.

MATERIALS AND METHODS

Cell culture. The K562 cell line (American Type Culture Collection) were grown in suspension and maintained (1 × 10^6 cells/ml) in RPMI 1640 tissue culture medium ( Gibco BRL, Life Technologies, Grand Island, NY), supplemented with 10% decomplemented bovine fetal serum (BFS). Cells were kept at 37°C in humid air (5% CO_2). K562 cells were observed as round bodies, with a high nucleus-to-cytoplasm ratio and a diameter of 13–23 μm. Human colon carcinoma T84 cells (American Type Culture Collection) were maintained in DMEM/F-12 media (GIBCO), supplemented with 10% BFS, at 37°C in humid air (5% CO_2).

RT-PCR. Total RNA was isolated using a SV Total RNA isolation system (Promega). Reverse transcription was performed for 60 min on 5 μg of total RNA from K562 cells by using moloney murine leukemia virus reverse transcriptase, oligo(dT)_12, primer, and 400 μM of each deoxyribonucleotide triphosphate (dNTP) at 42°C. PCR (30 cycles) at 94°C for 60 s, 58°C for 60 s, and 72°C for 60 s, followed by a final extension of 10 min at 72°C, was carried out using 5 μM of two specific oligonucleotide primers coding for nucleotides 4354–4374 and 4650–4630, respectively, of CFTR exon 24 (COOH terminus) sequence (sense 5’-ACTATTGCCGAAGC-CATT-3’, antisense 5’-CACCAGCACTCAAGCAAGTG-3’).

The human colon carcinoma cell line T84 was used as positive control (8, 47) and was processed as above. Control experiments were performed without the addition of reverse transcriptase to evaluate the absence of genomic DNA amplification.

Western blot analysis. K562 or T84 cells were washed with cold phosphate-buffered saline (PBS) and scraped in a small volume of lysis buffer (50 mM HEPES, 250 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, and 1 mM DTT). Nuclei and unbroken cells were removed with centrifugation (6,000 g for 15 min at 4°C). Total protein in each sample was quantitated using the bicinchoninic acid assay (BCA Protein, Pierce Chemical).

For Western blot studies, 100 μg of total proteins from K562 cells and 50 μg of total proteins from T84 cells were dissolved in loading buffer (4% sodium dodecil sulfate, 0.125 M Tris-HCl, pH 6.8, 0.2 M dithiothreitol, 0.02% bromphenol blue, and 20% glycerol). The preparation was heated to 90°C for 2 min, resolved on 7.5% polyacrylamide gel, and electrotransferred onto nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech, UK). These membranes were blocked with 1% (wt/vol) bovine serum albumin in PBS for 30 min at room temperature and incubated overnight with a mouse monoclonal antibody (Genzyme) against the R domain (MAb 13-1) of human CFTR diluted 1:1,000. Antibody specificity was demonstrated previously by Gregory et al. (16). Membranes were then washed with PBS-Tween 0.1% and incubated 1 h at room temperature with a goat anti-mouse IgG (Sigma Chemical) (1:1,000) conjugated with alkaline phosphatase. Filters were washed and revealed with BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Promega).

Immunoperoxidase studies. A few drops of the K562 cell suspension were placed on a slide and fixed in methanol. Imprints were rinsed in PBS and post-fixed permeabilized with absolute acetone for 30 min at −20°C. The cells were incubated in 1% BSA in PBS for 1 h at room temperature to quench nonspecific binding sites. The imprints were incubated for 1 h at 37°C with a mouse monoclonal primary antibody against R domain (MAb 13-1) of human CFTR (1:50 dilution) in a wet chamber. The samples were washed with PBS and incubated with an anti-IgG biotinylated anti-mouse antibody dilution 1:100 for 2 h at room temperature in a wet chamber. Staining was conducted with R.T.U. Vectastain kit (Vector Laboratories). Labeling was visualized by reaction with DAB (diaminobenzidine tetrahydrochloride) and counterstained with hematoxilin.

The samples were mounted on aqueous medium and observed under light microscopy. Non-immune mouse IgG without primary antibody was used as a negative control.

Electrical recordings. The activity of ion channels in K562 cells was recorded in the cell-detached (inside-out) configuration with a standard patch-clamp technology by using an amplifier Warner PC501 (Warner Instruments) with a 10 GΩ feedback resistor. Electrical signals were filtered at 2 KHz and digitized at 6 KHz using PCLAMP v.6 software (Axon Instruments, Union City, CA).

Forskolin (20 μM) was used to induce channel activation under cell-attached conditions, the patch was excised, and 100 μM CaM and 1 mM Mg-ATP were added to the bath to prevent channel rundown or inactivation (27, 37). Channel activity remained steady for 20–30 min.

In this study, 67% of the patches were multichannel patches and 33% were single-channel patches (n = 29). Event-duration and open probability histograms were constructed from records in which only a single open level was observed. Exponential functions were fitted using a least-squares routine of pCLAMP6. Single-channel records were further filtered at 750 Hz for display purposes only.

Solutions. Pipette and bath solutions contained (in mM) 140 NaCl, 5 KCl, 2.5 CaCl_2, 1 MgCl_2, and 10 HEPES (pH 7.4). For anion substitution experiments, NaCl in the bath was replaced by either Na-glucosinate or NaF. The catalytic subunit of the cAMP-dependent protein kinase (PKA) from Sigma was used at a final concentration of 10 μg/ml. Forskolin and glibenclamide from Alomone Labs (Jerusalem, Israel) and Sigma were diluted in DMSO and added directly to the chamber containing the cells in solution. DIDS from Sigma was dissolved in DMSO at a concentration of 20 mM and kept refrigerated until use. The final concentration of DMSO per se used did not affect ion channel activity (trials in our laboratory, with up to 0.2% DMSO on various cell types, data not shown).

The monoclonal antibody MAb 13-1 was directly diluted to 1:100 times in the bath solution from a stock solution (292 μg/ml). An inactivated antibody was obtained by preheating the CFTR antibody for 30 min at 100°C (22).

Calculations. To calculate the anion perm-selectivity ratio under F/Cl or gluconate/Cl asymmetrical conditions, current amplitudes were best fitted to the Goldman-Hodgkin-Katz equation (11)

\[
I(V_h) = \frac{x_i^2 F^2 P_i V_h [C_i/(1 - \exp(-|\alpha|))] + x_j^2 F^2 P_j V_h/RT \cdot [C_j/(1 - \exp(\beta))] }{C_i + C_j}
\]

where \( I \) is current amplitude, \( i \) (species in extracellular compartment) and \( j \) (species in intracellular compartment) represent Cl\(^-\) or other anion, depending on their locations on either side of the membrane, \( V_h \) the holding potential, is in mV, and \( z_i \) and \( z_j \) are the charges for species \( i \) and \( j \), respectively. \( P_i \) and \( P_j \) represent the permeability coefficients for}
the species $i$ or $j$, respectively, and $\alpha = z_i FV_i R T$, and $\beta = z_j FV_j R T$, where $R$, $T$, and $F$ have their usual meaning. We obtained ratios $P_{\text{gluconate}}/P_{\text{Cl}}$ and $P_{F}/P_{\text{Cl}}$ of 0.35 and 0.30, respectively (see Ref. 31 for limitations in this approach due to the nonindependence behavior of ions).

Data were expressed as means ± SD ($n =$ number of patches analyzed). For data comparisons, the Student’s $t$-test was performed. Differences were considered statistically significant when $P < 0.05$.

RESULTS

CFTR mRNA expression. To determine whether CFTR is expressed in the K562 cell line, total RNA was extracted and RT-PCR analysis was performed using primers to amplify exon 24 of human epithelial CFTR. T84 cell total RNA was used as positive control (47). An expected band of ~300 bp similar to positive controls was obtained (Fig. 1A). Similar results were observed in five other experiments. No product was detected in the absence of reverse transcriptase. The band was cloned and sequenced showing 100% identity to exon 24 of human CFTR (GenBank accession no. M28668).

Immunolocalization of CFTR in K562 cell membranes. Further determination of CFTR expression in K562 cells was conducted by Western blot analysis of the membrane proteins. Figure 1B shows blots of K562 cells. The membranes were incubated with a monoclonal antibody generated against exon 13 (R domain). A band of ~160 kDa was observed that corresponds to mature CFTR. In addition, another band of a lower molecular weight was evident in K562 cells representing an immature, deglycosylated form of CFTR. This is in agreement with previous results indicating that the immunoreactive bands in the 140- to 180-kDa regions represent various forms of the CFTR protein (35). The 160-kDa band was present in T84 cells used as a positive control. No bands were detected either in the absence of immune mouse serum or when the primary antibody was omitted (data not shown). Immunoperoxidase cytochemical labeling with the anti-CFTR antibody showed strong labeling of the plasma membrane of K562 cells (Fig. 2A). Control samples with nonimmune mouse IgG showed a very low staining (Fig. 2B) (4 experiments).

![Fig. 1. A: detection of cystic fibrosis transmembrane regulator (CFTR) mRNAs in K562 cells. Data shown are from lanes 1 to 4. One hundred-bp ladder, human colon carcinoma cells (T84) were used as a positive control; K562 and samples without reverse transcriptase were used as a negative control. B: immunoblots for CFTR in membrane extracts from K562 cells. Membranes were incubated overnight with a mouse monoclonal antibody against the R domain (MAb 13-1) of human CFTR. A band between 150 and 160 kDa was seen corresponding to CFTR protein. In addition, another band of a lower molecular weight was evident in K562 cells, consistent with an immature, deglycosylated form of CFTR. The 160-kDa band was present in T84 cells used as a positive control.](http://ajpcell.physiology.org/)

![Fig. 2. Localization of CFTR protein in K562 cells by immunoperoxidase (A and B) using anti-CFTR antibody against R domain. K562 cells were incubated with a mouse monoclonal primary antibody against R domain (Mab 13-1) of human CFTR. The samples were washed and then incubated with an anti-mouse antibody, anti-IgG biotinylated. CFTR labeling was visualized by diaminobenzidine tetrahydrochloride (DAB) reaction and counterstaining with hematoxilin. A strong labeling was observed in the cell membranes (A). Replacement of primary antibody with nonimmune IgG showed a very low staining (B). Magnification, ×1,000.](http://ajpcell.physiology.org/)
Electrical activity of K562 cells. Forskolin (20 μM), an adenylyl cyclase activator, was used to induce channel activation under cell-attached conditions. Channel activity appeared 2–3 min after addition of the drug to the extracellular solution. After that, the patch was excised and the catalytic subunit of PKA (10 μg/ml) and 1 mM Mg-ATP were added to the bath. Channel activity remained steady for 20–30 min. Figure 4 shows one such experiment in which ion channel activity in patches held at 80 and 60 mV was not affected by PKA and ATP added to the cytoplasmic side of the patch. In six experiments, a comparison of the open probability (P_o) values for single channels obtained in the presence or absence of PKA plus ATP (0.44 ± 0.09 vs. 0.36 ± 0.09, n = 6; P < 0.3) indicated no differences for addition of the enzyme.

To further demonstrate the anionic selectivity of this channel, anion substitution experiments were also performed. NaCl (140 mM) in the bath was replaced by either 140 mM Na-glucuronate or 140 mM NaF. All other constituents of the solution were similar; thus substitution solutions contained 12 mM residual Cl⁻. Figure 5A shows single channels recorded in excised, inside-out patches held at different positive and negative potentials with either Cl⁻ or gluconate on the intracellular side (Fig. 4A). The I-V relationship determined in these substituted solutions with gluconate or F⁻ are shown in Fig. 5B. Currents could be observed, although they were smaller at negative potentials and under cell-attached conditions was sufficient to maintain a high level of activation even after excision. Thus further addition of PKA and ATP to excised patches would result in no change in the already active channel function. The experimental protocol was as follows: forskolin (20 μM) was used to induce channel activation under cell-attached configuration in otherwise quiescent patches. Ion channel activity became apparent 2–3 min after the addition of the drug to the extracellular solution. After that, the patch was excised and the catalytic subunit of PKA (10 μg/ml) and 1 mM Mg-ATP were added to the bath. Channel activity remained steady for 20–30 min. Figure 4 shows one such experiment in which ion channel activity in patches held at 80 and 60 mV was not affected by PKA and ATP added to the cytoplasmic side of the patch. In six experiments, a comparison of the open probability (P_o) values for single channels obtained in the presence or absence of PKA plus ATP (0.44 ± 0.09 vs. 0.36 ± 0.09, n = 6; P < 0.3) indicated no differences for addition of the enzyme.

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channel activity in excised patches of K562 cells (Fig. 6). Figure 6 (right) also shows histograms of current amplitude in control, heat-inactivated, and active MAb 13-1. In the presence of the active antibody, the open level distribution is shifted towards the closed level (C).

The effect of the CFTR blocker glibenclamide was also assessed. Figure 7A shows a representative record in which ion channel activity was reduced by glibenclamide (100 μM) added to the cytoplasmic side of the patch. Glibenclamide had no effect on single-channel amplitude but increased the frequency of open-to-closed transitions. This effect was not markedly dependent on the membrane potential (Fig. 7B, n = 6). The effect of the drug was also observed in the open and closed-time histograms (Fig. 8). A minimum resolution of 1 ms was imposed on the data by ignoring all events shorter than this time (12). Addition of glibenclamide caused a reduction in the open time constant (Fig. 8, left) from 23.2 ± 7.1 to 2.1 ± 1.4 ms (n = 7) for control and test solution, respectively (P < 0.01). No effect on the closed time constant was observed: 39.3 ± 16.3 vs. 42.0 ± 22.6 ms (n = 7, P = 0.2). The effect of different glibenclamide concentrations was also assessed (25, 50, and 100 μM), indicating that the decrease in $P_o$ was concentration dependent, with a half-maximum inhibitory ($K_i$) of 42.3 ± 4.6 μM (n = 3, SD). The effect of DIDS, another CFTR blocker (26, 33, 40), was also assessed in inside-out patches with stable channel activity (Fig. 9). As with glibenclamide, addition of DIDS (200 μM) had no effect on single-channel amplitude but decreased $P_o$ from 0.55 ± 0.23 to 0.13 ± 0.09 for control and test solutions, respectively (n = 4, P < 0.01). The effect of DIDS was irreversible. Even when the patch was washed several times with control solution, the $P_o$ did not recover (Fig. 9) with a value of 0.09 ± 0.01 not different from that obtained in the presence of the drug (n = 4).

DISCUSSION

In this study, we determined the presence and functional characterization of a Cl$^-$ channel in K562 human leukemia cells. We found a cAMP-activated, non-rectifying anion channel that is inhibited by glibenclamide, DIDS, and anti-CFTR antibodies. RT-PCR and Western blot determined the presence of the CFTR gene product, and protein expression was also confirmed by immunohistochemistry.

Our data indicate that forskolin addition under cell-attached conditions was sufficient to maintain a high level of activation even after excision. Thus further addition of PKA and ATP to excised patches would result in no change in the already active channel function. This, however, does not indicate the lack of PKA responsiveness; rather, the effect of supramaximal concentration of forskolin was sufficient to fully activate the channels. The lack of effect of added PKA in K562 cells is most consistent with experimental conditions under which rundown of forskolin-stimulated CFTR activity is very slow or practically absent in the excised patches without PKA.
CFTR is normally activated in cells by cAMP signaling pathways. The cAMP-dependent PKA phosphorylation event activates the channel, which in turn can be inactivated by phosphatases (1; see Ref. 17 for a review). Thus it is possible that PKA was not required in our experiments because little phosphatase activity was present in the patches. This interpretation was raised by Sørensen and Larsen (40), who reported that no rundown of CFTR channel activity in inside-out patches was noted in the presence of ATP after removing cAMP-dependent protein kinase from the solution. The same interpretation is given by Luo et al. (27) in patches from transfected Chinese hamster ovarian cells.

Another possibility to be considered is that after the excision of the patch, the addition of cAMP could indeed activate PKA bound to the membrane. This is consistent with Bradbury (6), who shows a physical association between CFTR and PKA and that in T84 cells PKA subunits are cytosolic (RI) and membrane bound (RII). Earlier evidence has also shown that PKA is found in the membranes of red blood cells (see Ref. 42).

The Cl\(^-\) channels described in this report had a single-channel conductance and an anion perm-selectivity consistent with CFTR. Conductance values reported for CFTR have a range of 6–15 pS, and subconductance states have been observed under certain experimental conditions (15, 46). Some variations in the reported conductance and kinetics are the consequence of variable parameters, including recording conditions, species-dependence, and ATP concentrations. Differences between wild-type and transfected CFTR have also been observed (13, 14, 23, 27, 36, 39, 52, 54). In this study, the gluconate/Cl and F/Cl permeability ratios for these channels were studied under bi-ionic conditions. Cl\(^-\) replacement by either F\(^-\) or gluconate shifted the E\(_m\) to more negative potentials. Both ionic substitutions exhibited relative perm-selectivity ratios of ~0.3. Similar perm-selectivity ratios for these anions were described by others for CFTR expressed either endogenously or heterologously in a number of cell types (reviewed in Ref. 31). In fibroblasts infected with a retrovirus expressing CFTR, for example, F\(^-\) stimulated open-state probability of CFTR channels in the presence of ATP after phosphorylation (5). When gluconate or F\(^-\) were the main anions in the bath, the current amplitude was smaller at negative potentials and greater at positive potentials (Fig. 5). This is consistent with previous findings in Chinese hamster ovarian cells (45) expressing wild-type CFTR when bathing Cl\(^-\) was replaced by F\(^-\). In our own study, the higher current amplitude at positive potentials when Cl\(^-\) was replaced as the main anion could be explained if we had subconductance states, with lower amplitude current levels being more evident in the control solution. Although interesting, this possibility is unlikely because we did not observe subconductance levels under any of our experimental conditions.

CFTR channels are characterized by their sensitivity to blocking by several agents, including the sulfonilurea hypoglycemic agent glibenclamide and its congeners tolbutamide (reviewed in Refs. 19, 53). The blocking effect of glibenclamide in excised membrane patches seen in the present study had a K\(_i\) value of 42 \(\mu\)M, in agreement with earlier observations (13, 14, 36, 39). These studies suggest an open-channel blockade and the drug gaining access to its binding site in the channel from the intracellular side of the membrane (see also Ref. 1). Open time constant of channels exposed to glibenclamide decreased, in agreement with...
data from Schultz et al. (36) in mouse L cells and those of Sheppard et al. (39) obtained in mammalian epithelial cells. Our results showed a weak voltage dependence for the glibenclamide effect, with the effect being more evident at negative potentials (Fig. 7B). We also found that DIDS caused an irreversible block of the channels when added to the cytosolic side of inside-out patches, supporting previous results by Sørensen et al. (40) in the luminal membrane of frog skin exocrine gland acini. Linsdell and Hanrahan (26) also reported that, when added to the inside, DIDS blocked whole CFTR currents recorded from excised patches of transfected Chinese hamster ovarian cells. Similar results were obtained by Reddy and Quinton (33) in the human sweat duct.

Active transcription translation of the CFTR gene product is detectable in a variety of epithelial and nonepithelial cells (49, 51). These observations of CFTR widespread distribution suggest a housekeeping gene (51), implying a great range of functions in many human blood cells, including possible regulatory roles in the secretion of antibodies and cytokines by lymphocytes in response to extracellular signals (9, 30, 44) and regulation of lipopolysaccharide and interferon-γ-induced macrophage activation (18). In K562 cells, CFTR...
and other channels could be involved in various cell functions, including control of the membrane potential and cell differentiation and proliferation (10, 24, 48). Previous studies demonstrated that ion channels have specific functions in K562 cells. Thus Sheehy (38) found an increment in ion channel activity in human lymphocytes by lymphokines. A selective upregulation of human ether-a-go-go-related gene (HERG) K channels has also been reported in neoplastic hematopoietic cells, including K562 cells, providing a marker and target for potential therapeutics (41). In conclusion, our data demonstrated the presence of a cAMP-activated channel, nonrectifying anion channel highly selective for Cl⁻ over Na⁺ and with gluconate/Cl and F/Cl perm-selectivity ratios of about 0.3. This is most consistent with the presence of a functional CFTR in K562 cells, including cell functions such as differentiation and proliferation.

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**DISCLOSURES**

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