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Protein kinase C mutants in the auto-inhibitory region exhibit two distinct properties

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To define the role of the auto-inhibitory region of protein kinase C (PKC), Arg[22]-Lys[23]-Gly[24]-Ala[25]-Leu[26]-Arg[27], site-directed mutations were introduced into the basic residues. Three mutants, PKC^{Ala22,23}, PKC^{Ala27}, and PKC^{Ala22,23,27}, apparently fell into two distinct types with regard to their biochemical properties and biological activities, as judged by the enhancement of a c-*fos* promoter in Jurkat cells and by the initiation of germinal vesicle breakdown (GVBD) in *Xenopus laevis* oocytes. (i) PKC^{Ala22,23} and PKC^{Ala27} had activators independent in vitro kinase activity, high phosphorylation levels in vivo, and localized in both cytosolic and particulate fractions. These mutants were not fully biologically active. (ii) PKC^{Ala22,23,27} had a low phosphorylation level in vivo, was found predominantly in the particulate fraction and was the most biologically active. These results suggest that basic residues in the auto-inhibitory domain account for the regulation of kinase activity and the cytosolic retention of PKC. The particulate association or the cytosolic clearance of PKC may facilitate signal transduction in the cell.

Protein kinase C; Auto-inhibitory region; Mutation

1. INTRODUCTION

Protein kinase C (PKC) is a key enzyme involved in the regulation of various cellular responses, such as cell growth, development, tumor promotion, secretion and gene regulation (see [1] for a review). The molecular mechanism by which PKC activity is regulated in the cell is an important issue due to its broad biological effects. Earlier studies on PKC have shown that limited proteolysis of this enzyme generates a fragment which carries the phorbol ester binding activity and a fragment that is catalytically active [2,3], indicating that PKC is composed of regulatory and catalytic domains. Cloning of various PKC cDNAs has revealed that putative kinase and regulatory domains can be found in all members of the PKC family (see [4] for a review). Consistant with this notion, it has been shown that phorbol ester binds to the N-terminal regulatory domain [5], and the catalytic domain has been shown to be sufficient to induce PKC-mediated cellular responses without phorbol ester [6].

A general mechanism for the regulation of protein kinase activity by an auto-inhibitory domain has been proposed for various protein kinases (see [7] for a review), including myosin light chain kinase [8,9], calcium/calmodulin-dependent protein kinase II [10–12], cAMP-dependent protein kinase [13], and PKC [14]. In the case of PKC, the existence of such a built-in inhibitor was first suggested when a synthetic peptide containing the substrate-like motif, Arg[22]-Lys[23]-Gly[24]-Ala[25]-Leu[26]-Arg[27], was found to be a potent competitive inhibitor [14]. The proposed scheme is that the interaction of activator of PKC with the regulatory domain induces a conformational change that relieves the pseudo-substrate inhibition and elicits catalytic activity. Mutation studies using a series of truncated PKC mutants [15] or a site-directed mutant [16] have shown that alterations to the auto-inhibitory region lead to activity. In order to further define the role of the auto- inhibitory region of PKC, we generated mutant PKC with site-directed mutations in this region. In this study, basic residues found in the auto-inhibitory region were particulary targeted for mutations, since basic residues in the analogus positions are usually the critical determinants for PKC substrates [17,18].

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2. MATERIALS AND METHODS

2.1. *Construction of mutant PKC cDNA expression vectors*

Three different mutant PKCα cDNAs were constructed using bovine PKCα cDNA [19] and the following eight synthetic oligonucleotides:

CPM1, 5′-CGCCGCAGGCGTCGCCAACCGCTTCGCCCGCA-AAGGGGCG-3′

CPM2, 5′-CAGCGCCCCTTTGCGGGCGAAGCGGTTGGCGA-CGTCCTGCGG-3′

CPM3, 5'-CTGAGGCAGAAGAACGTGCACGAGGTGAAGA-ACCACCGCTTCATCG-3'

CPM4, 5'-CGCGCGATGAAGCGGTGGTTCTTCACCTCGTG-CACGTTCTTCTGCCT-3'

CPM5, 5'-CGCCGCAGGACGTCGCCAACCGCTTCGCCGCT-GCTGGGGCG-3'

CPM6, 5'-CAGCGCCCCAGCAGCGGCGAAGCGGTTGGCG-ACGTCCTGCGG-3'

CPM7, 5'-CTGGCTCAGAAGAACGTGCACGAGGTGAAGA-ACCACCGCTTCATCG-3'

CPM8, 5'-CGCGCGATGAAGCGGTGGTTCTTCACCTCGTG-CACGTTCTTCTGAGC-3'

CPM1 and CPM2, CPM3 and CPM4, CPM5 and CPM6, CPM7 and CPM8 were annealed after phosphorylation to make the double-stranded DNA fragments, CPMA, CPMB, CPMC and CPMD, respectively. CPMA and CPMD, CPMB and CPMC, and CPMC and CPMD were ligated into the *Nar*I–*Bss*HIII site of pSRα-PKCα [6,20] to produce, pSRα-PKC^{Ala27}, pSRα-PKC^{Ala22,23} and pSRα-PKC^{Ala22,23,27}, respectively (Table I, columns 1 and 2). One base pair change generating an *Aat*II site without affecting the amino acid sequence was introduced into the oligonucleotides to aid in the screening procedure. The inserted oligonucleotide portions were verified by sequencing using Sequenase (US Biochem. Corp.).

2.2. *Immunoblotting analysis and [^{3}H]phorbol 12,13-dibutylate (PDBu) binding activity of the mutant PKCs*

Immunoblotting analysis was performed as described [15] using PKC the monoclonal antibody, MC-5 (Amersham). Soluble and particulate fractions were prepared by the following procedure. COS7 cells (1×10^6) were transfected by the DEAE-dextran method [20] with 5 μg of pSRα vector, pSRα-PKC, pSRα-PKC^{Ala22,23}, pSRα-PKC^{Ala27}, or pSRα-PKC^{Ala22,23,27}. Cells were harvested 48 h after transfection, suspended in 100 μl of buffer A (20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2 mM EGTA, 0.25 M sucrose, 2 mM 2-mercaptoethanol, and 100 μM phenylmethylsulfonyl fluoride), and sonicated for 20 s. Cell homogenates were centrifuged at $10^5 \times g$ for 1 h and the supernatants were used as soluble fractions. The pellets were solubilized in 100 μl of buffer A containing 1% SDS and the supernatants, following centrifugation under the same conditions stated above, were used as particulate fractions. [^{3}H]PDBu binding was performed as described previously [6].

2.3. *In vitro kinase activity of the mutant PKCs*

In vitro kinase assays were performed as previously described [15] with the following modifications. Briefly, COS7 cells (1×10^6/10 cm plate × 3) transfected with mutant PKC plasmids were harvested and homogenized in 0.45 ml of ice-cold buffer A containing 0.5% Triton X-100 and sonicated for 20 s. Cell homogenates were centrifuged at $10^5 \times g$ for 1 h. The supernatants were adsorbed on DEAE-Sephacel columns (0.5 ml) and eluted stepwise with buffer A containing 90 mM and 200 mM NaCl. Protein kinase activity was measured using histone H1 as substrates. The reaction mixture (100 μl) contained 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.25 mM $CaCl_2$, 0.1 mM EDTA, 0.1 mM EGTA, 10 μM [γ-^{32}P]ATP (1,000–3,000 cpm/pmol), 4 μg/ml phosphatidylserine, homogenate, and 10 μM histone H1 or 100 mM EGF-R peptide. After incubation for 10 min at 30°C, a 40 μl aliquot was removed from the reaction mixture and applied to P-81 paper (Whatman); free [γ-^{32}P]ATP was removed by washing with 75 mM H_3PO_4.

2.4. *The level of phosphorylation of the mutant PKCs in vivo*

Immunoprecipitation of PKC was performed in the following manner. Transiently transfected COS7 cells (1×10^6) were labeled with 1 mCi of [^{32}P]orthophosphate (Amersham) or Tran-[^{35}S] (ICN) for 12 h. Cell homogenates were prepared in 100 μl of buffer A supplemented with 1% SDS and centrifuged at $10^5 \times g$. 100 μl of buffer B (40 mM Tris-HCl, pH 7.5, 10 mM EDTA, 2% Triton X-100, 2% sodium deoxycholate, 5 mg/ml bovine serum albumin, 2 mM Na_2MoO_4, 100 μM phenylmethylsulfonyl fluoride) and 5 μg of MC-5 monoclonal antibody were added to 50 μl of each cell homogenate and gently shaken at 4°C for 6 h. Immunocomplex was removed by adding protein G–agarose and washing several times with buffer B without BSA. Finally, the pellets were dissolved in 15 μl of Laemmli sample buffer, boiled and electrophoresed on a 7.5% SDS-polyacrylamide gel.

2.5. *Bioassays for PKC activity in cells*

Transfections for Jurkat cells and luciferase assays were performed as previously described [20]. The c-*fos*–luciferase plasmid carries 0.4 kb of the 5' flanking region of the c-*fos* gene [21]. Injection of mutant PKC plasmids and the assay for germinal vesicle breakdown (GVBD) in *Xenopus laevis* oocytes were performed as described [6]. Briefly, a total of 0.01–10 ng of PKC plasmid DNA in 10 nl of distilled water was injected into the nucleus of each oocyte. 7–10 oocytes were used per sample and observation was made between 12 and 18 h after injection. Initiation of the GVBD was judged by the appearance of a white spot at the animal pole, and the frequency was calculated. pSRα-PKAC [6], which lacks the coding region for the regulatory domain and thus is constitutively active, was used as a positive control for these biological assays.

3. RESULTS

3.1. *Expression of mutant PKCs in COS7 cells and phorbol ester binding*

The biochemical properties of the mutant PKCs were assessed in a COS7 cell over-expression system, as described in Materials and Methods. Transfection with either wild-type or mutant PKC cDNA plasmids yielded a 6–9-fold increase in immunoreactive PKC over the endogenous level (Table I, column 3). Proteolytic products were not detected for either the wild-type or the mutant PKCs (see Fig. 1). In examining phorbol ester binding of the mutant PKCs, approximately a 10-fold increase in [^{3}H]PDBu binding was detected in COS7 cells expressing the wild-type or the mutant PKC (Table 1, column 4). Therefore, the mutations in the pseudo-substrate region did not impair the phorbol ester binding activity.

3.2. *Subcellular localization of mutant PKCs*

The subcellular localization of wild-type and mutant PKCs was investigated (Fig. 1). While endogenous PKC was found primarily in the soluble fraction which is extracted from the cytosol by divalent cation chelators, wild-type PKC expressed from transfected cDNA was distributed in both the soluble and particulate fractions. This indicates that half of the wild-type PKC product in this expression system is trapped in the particulate fraction. PKC^{Ala22,23} and PKC^{Ala27} were also found in both the soluble and particulate fractions, in a similar ratio as that observed for wild-type PKC. In contrast, PKC^{Ala22,23,27} was found primarily in the particulate fraction with only a small amount in the soluble fraction. This suggests that PKC^{Ala22,23,27} is more tightly

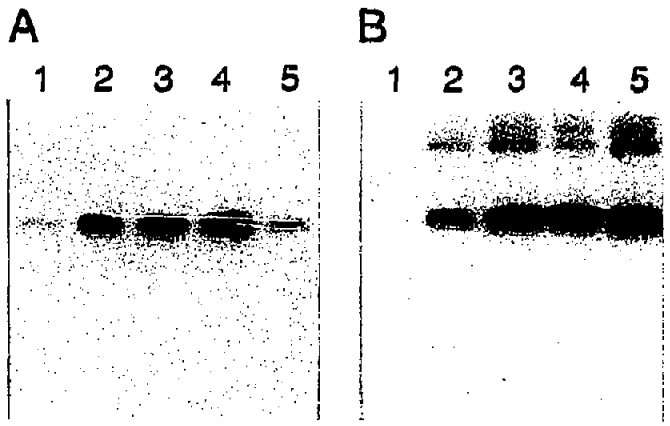

Fig. 1. Subcellular localization of wild-type and mutant PKCs. Wild-type and mutant PKCs in the soluble (panel A) and the particulate (panel B) fractions from COS7 cells were detected by immunoblotting analysis. Samples were prepared from cells transfected with pSRα vector (lane 1), pSRα- PKC (lane 2), pSRα-PKC^{Ala22,23} (lane 3), pSRα-PKC^{Ala27} (lane 4), and pSRα-PKC^{Ala22,23,27} (lane 5).

associated with the particulate fraction than wild-type PKC, PKC^{Ala22,23} or PKC^{Ala27}. In an immunocytochemical study using indirect immunofluoresence, wild-type PKC, PKC^{Ala22,23} and PKC^{Ala27} showed a diffuse cytosolic staining (data not shown). Only PKC^{Ala22,23,27} was hardly detected in the cytosol by the same method, suggesting that it may have a different localization in the cell or that the epitope of this mutant is masked.

3.3. *In vitro kinase assay of the partially purified mutant PKCs*

Wild-type and mutant PKCs expressed in COS7 cells were partially purified and kinase activity was determined as described in Materials and Methods (Table II). Wild-type PKC expressed in COS7 cells was collected in the 90 mM NaCl fraction and gave a 4-fold increase in activator-dependent kinase activity compared to endogenous PKC. PKC^{Ala22,23} and PKC^{Ala27} primarily eluted in the 200 mM NaCl elution, rendering a high level of activator-independent kinase activity in this fraction. Its kinase activity was only slightly increased (1.5-fold) by the addition of the activators. A slight increase in kinase activity was also observed in the 90 mM fraction, suggesting that additional PKC^{Ala22,23} and PKC^{Ala27} eluted in this fraction. PKC^{Ala22,23,27} was much more refractory to solubilization, as described in the former section, and very little amount of soluble PKC^{Ala22,23,27} could be obtained. Therefore its kinase activity could not be directly compared with the wild-type or other mutant PKCs. Since expression of PKC^{Ala22,23,27} did not confer an increase in kinase activeity on the particulate fraction (data not shown), it is unlikely that PKC^{Ala22,23,27} has a high level of kinase activtiy.

Table II

In vitro activity of mutant PKCs expressed in COS7 cells

Transfected mutant PKC cDNA	99 mM eluate (×10^3 cpm[a])		200 mM eluate (×10^3 cpm[a])	
	EGTA	PS+Ca+TPA	EGTA	PS+Ca+TPA
pcDSRα vestor	2.5	12.5	4.3	9.5
PKC (wild type)	3.5	49.2	7.7	9.1
PKC^{Ala22,23}	9.1	15.6	105.1	146.0
PKC^{Ala27}	15.2	26.2	122.9	182.8

[a] ^{32}P incorporated in histone H1 as described in Materials and Methods

3.4. *The level of phosphorylation of the mutant PKCs in COS7 cells*

COS7 cells transfected with wild-type or mutant PKC plasmids were labeled with [^{35}S]methionine or [^{32}P]orthophosphate for 12 h and immunoprecipitation was performed using the monoclonal antibody, MC-5 (Fig. 2A and B). Wild-type and mutant PKCs expressed in COS7 cells were detected as two bands of 74 kDa and 77 kDa, in approximately the same amount (Fig. 2A). While phosphorylation of endogenous PKC could not be detected (Fig. 2B, lane 1), a low level of phosphorylation was detected for the wild-type PKC expressed in the cell (Fig. 2B, lane 2). The phosphorylated PKC band

Table I

PKC mutants in the auto-inhibitory region and their expression and PDBu binding in COS7 cells

Transfected mutant PKC cDNA	Coding sequence of the auto-inhibitory region	^{125}I count of the immunoblot band × 10^3 cpm (relative amount)	[^{3}H]PDBu binding per 10^6 cells ×10^3 cpm (relative amount)
pcDSRαvector		6.08(1.0)	11.2 (1.0)
PKC (wild-type)	Arg^{22}-Lys-Gly-Ala-Leu-Arg	34.5 (5.7)	90.9 (8.1)
PKC^{Ala22,23}	Ala-Ala-Gly-Ala-Leu-Arg	157.2 (9.4)	150.9 (13.5)
PKC^{Ala27}	Arg-Lys-Gly-Ala-Leu-Ala	148.9 (8.0)	140.4 (12.5)
PKC^{Ala22,23,27}	Ala-Ala-Gly-Ala-Leu-Ala	148.3 (7.9)	113.3 (10.1)

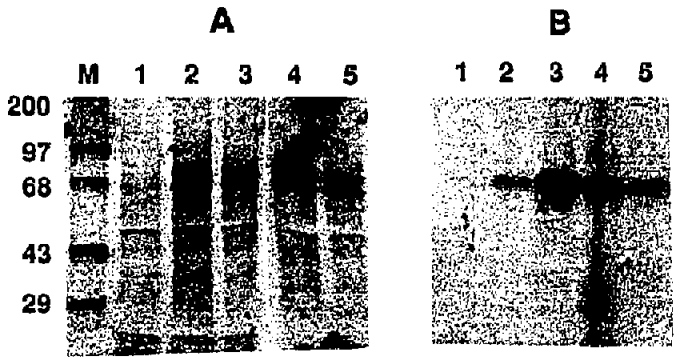

Fig. 2. Phosphorylation of wild-type and mutant PKCs in vivo. Immunoprecipitation was performed on extracts from cells labelled with [^{35}S]methionine (panel A) or [^{32}P]orthophosphate (panel B) from cells transfected with pSRα vector (lane 1), pSRα-PKC (lane 2), pSRα-PKC^{Alu22,23} (lane 3), pSRα-PKC^{Alu27} (lane 4) and pSRα-PKC^{Alu22,23,27} (lane 5). Lane M, molecular weight markers.

corresponded to the upper 77 kDa band. Phosphorylation levels were significantly higher for PKC^{Alu22,23} and PKC^{Alu27} than for the wild-type PKC. In contrast, PKC^{Alu22,23,27} had a lower phosphorylation level similar to that of the wild-type PKC.

3.5. *Activation of c-fos promoter and initiation of GVBD by the mutant PKC plasmids*

The biological activity of the mutant PKCs was examined in two independent assays which have been described previously [6]. The mutant PKC cDNA plasmids were transfected into Jurkat cells with a c-*fos*–luciferase reporter gene (Fig. 3A). The c-*fos*–luciferase gene had a low basal expression level in Jurkat cells and was not activated by co-transfection with pSRα-PKC, demonstrating that wild-type PKC is inactive by itself. Co-transfection with pSRα-PKC^{Alu22,23} and pSRα-PKC^{Alu27} did not activate the c-*fos*–luciferase gene either. In contrast, transfection with pSRα-PKC^{Alu22,23,27} significantly enhanced expression of the c-*fos*–luciferase gene. The level of enhancement was comparable to that of pSRα-PKAC, which lacks the whole regulatory domain and thus is constitutively active [6].

The biological activity of the mutant kinases were characterized by another assay employing *Xenopus laevis* oocytes which induce germinal vesicle breakdown (GVBD) after the addition of phorbol esters [22]. This oocyte system provides a sensitive bioassay to detect constitutively active PKC, in vivo [6]. None of the oocytes injected with pSRα-PKC plasmid induced GVBD, indicating that wild-type PKC is inactive in oocytes (Fig. 3B). In contrast, 80% of the oocytes injected with the positive control, pSRα-PKAC, initiated GVBD in the absence of phorbol ester. Among the mutant PKC plasmids, injection of pSRα-PKC^{Alu22,23,27} gave the highest initiation rate of GVBD, comparable to that observed with pSRα-PKAC. Injection of pSRα-PKC^{Alu22,23} or pSRα-PKC^{Alu27} also initiated GVBD but

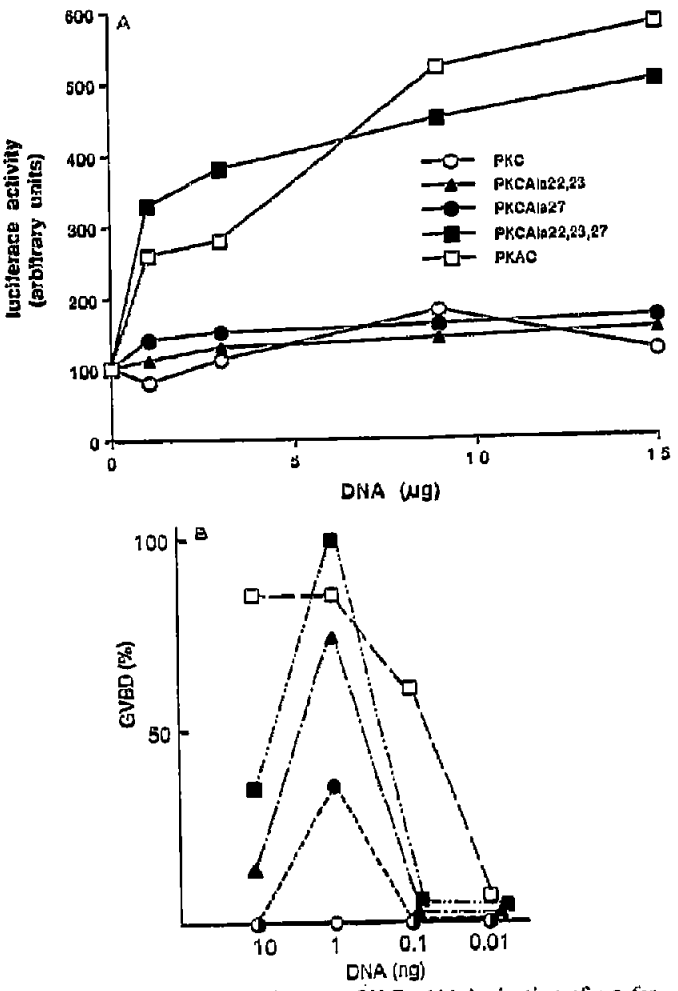

Fig. 3. Biological activity of mutant PKCs. (A) Activation of a c-*fos*–luciferase gene in Jurkat cells by expressing mutant PKCs. (B) Initiation of GVBD in *Xenopus laevis* oocytes by expressing mutant PKCs.

at a lower frequency. Injection of higher concentrations of mutant PKC plasmids appeared to interfere with the GVBD process.

4. DISCUSSION

By introducing 'changed-to-alanine' mutations into the basic residues in the auto-ihibitory region of PKC, mutants with different biological and biochemical properties were generated. PKC^{Alu22,23} and PKC^{Alu27} had a high level of activator-independent in vitro kinase activity, indicating that substitution of one or two basic residues are sufficient to distroy the pseudo-substrate inhibition. The tight dependence of this inhibition to these basic residues suggests that this region might be interacting with the catalytic center by a mechanism similar to the actual substrate. These mutants with high catalytic activity localized in both cytosolic and particulate fractions, indicating that particulate association does not correlate with in vitro kinase activity or in vivo

phosphorylation. The other mutant, $PKC^{Ala22,23,27}$, was tightly associated with the particulate fraction. After phorbol ester treatment of COS7 cells, endogenous PKC was not found in the cytosolic fraction but was only detected in the particulate fraction, indicating that PKC underwent translocation (data not shown). Likewise, the association of $PKC^{Ala22,23,27}$ with the particulate fraction might reflect a similar translocational event that occurs following phorbol ester treatment. It is unlikely, although it cannot be ruled out, that the increase in the hydrophobic residues in the auto-inhibitory region accounts for the particulate association, since the hydropathic profile of $PKC^{Ala22,23}$ and $PKC^{Ala22,23,27}$ did not show a significant difference. Whatever is the cause of the difference in the localization, the proper structure of the auto-inhibitory region is needed for the cytosolic retention of PKC.

High levels of kinase activity per se, as demonstrated in $PKC^{Ala22,23}$ and PKC^{Ala27}, was insufficient to elicit full biological activity after expression. Instead, $PKC^{Ala22,23,27}$, which was most tightly associated with the particulate fraction, was much more biologically potent. Although in vitro kinase activity of $PKC^{Ala22,23,27}$ could not be rigorously demonstrated, we assume that it had enough kinase activity to elicit biological responses, and, by translocating to the particulate fraction, it facilitated the response by tightly coupling with the target proteins. On the other hand, the lower biological potentials of $PKC^{Ala22,23}$ and PKC^{Ala27} can be explained by their inefficiency of access to the target proteins. On the other hand, it may also be possible that clearlance of active PKC from the cytosol may be required to elicit a biological response [23]. These mutant PKCs with different biological and biochemical properties may be useful tools to investigate the signal transduction events involving PKC in the cell.

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