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ACTIVATION OF PKN, A NOVEL 120-KDa PROTEIN KINASE WITH LEUCINE ZIPPER-LIKE SEQUENCES,BY UNSATURATED FATTY ACIDS AND BY LIMITED PROTEOLYSIS

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Summary: PKN, a novel protein kinase with catalytic domain homologous to PKC family and unique amino terminal leucine zipper-like sequences,was purified partially from COS7 cells transfected with the cDNA construct encoding human PKN for enzymatic characterization of the enzyme. Using serine containing synthetic peptides based on PKC pseudosubstrate sites as the phosphate acceptors, kinase activities estimated from partially purified PKN were not stimulated by Ca^{2+}/phosphatidylserine/diolein but were activated several-fold to several tens-fold by 40μM unsaturated fatty acids, such as arachidonic acid, linoleic acid, and oleic acid. Autophosphorylation of the immunoprecipitates using anti-PKN antiserum was also stimulated by various unsaturated fatty acids. Limited proteolysis of PKN with trypsin induced an enhancement of the peptide kinase activity that was almost independent of arachidonic acid. © 1994 Academic Press, Inc.

Recently we have described the primary structure of a novel protein kinase, designated PKN, with catalytic domain homologous to PKC family and unique N-terminal leucine zipper-like sequences (1). PKN is a ubiquitously expressed serine/threonine kinase, physiological substrates have not been identified and enzymatic property and its regulation have not yet been determined. During the course of studying the enzymatic property of PKN, we found that the enzyme was significantly activated by unsaturated fatty acids or protease.

MATERIALS AND METHODS

Materials: Construction of the expression plasmid for human PKN cDNA was reported previously (1). Calmodulin was purified from rat brain using a phenyl-Sepharose column, as described (2). S6 peptide (corresponds to amino acid 231-239 of human 40S ribosomal protein S6; RRRLSSLRA)

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The abbreviations used are: PS, phoshatidylserine; N-terminal, amino terminal; MBP, myelin basic protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DTT, dithiothreitol; C-terminal, carboxyl terminal; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ssDNA, single stranded DNA.

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was purchased from Santa Cruz Biotechnology, Inc. The following oligopeptides were synthesized with an automated peptide synthesizer (Applied Biosystems, model 430A): αPKC peptide (corresponds to the amino acid 15-31, substituting Ser for Ala; DVANRFARKGSLRQKNV); δPKC peptide (corresponds to the amino acid 137-153, substituting Ser for Ala; AMFPTMNRRGSIKQAKI); εPKC peptide (corresponds to the amino acid 149-164, substituting Ser for Ala; ERMRPRKRQGSVRRRV); ζPKC peptide (corresponds to the amino acid 109-125, substituting Ser for Ala; GEDKSIYRRGSRRWRKL). Other chemicals were obtained from commercial sources.

Expression of PKN in COS7 cells and purification: COS7 cells ($1x10^7$) were transfected with 75μg of PKN cDNA-construct or control construct (PKN cDNA was ligated to pTB701 expression vector (3) in reverse order, which was defined as "control construct") using Lipofectamine (Gibco BRL). Cells were harvested after 72 hr incubation at 37°C. The following procedures were carried out at 0-4°C. The cells were homogenized in 4 vols. of homogenization buffer by Teflon/glass homogenizer (15 strokes). Homogenization buffer contained 50 mM Tris/HCl, pH 7.5, 5 mM EGTA, 5 mM EDTA, 0.02% NaN_3, 0.5 mM DTT, 10 μg/ml leupeptin, and 1 mM PMSF. After centrifugation (20000 x g for 1h), solid ammonium sulfate was added to the supernatant to obtain 20 % saturation . The solution was left standing with stirring for 30 min and centrifuged at 15000 x g for 20 min. Solid ammonium sulfate was added to the supernatant to obtain 40 % saturation and recentrifuged, then resulting pellet was dissolved in buffer A (50 mM Tris/HCl, pH 7.5 containing 1mM EGTA, 1 mM EDTA, 0.02 % NaN_3, 0.5 mM DTT, and 0.2 μg/ml leupeptin). This sample was loaded onto a 10 ml of Bio-Gel P-6 desalting gel (Bio-Rad), and eluted with buffer A. The desalted sample was applied onto 1 ml Resource Q column (Pharmacia) preequilibrated with buffer A, which was connected to a Pharmacia FPLC system. The column was then washed with 20 ml of buffer A containing 50 mM NaCl, and the sample was eluted by application of a 20-ml linear concentration gradient of NaCl (50-1500 mM) in buffer A. Fractions of 1ml each were collected.

Kinase assay: Five μl of Resource Q fraction #4 was incubated for 5 min at 30°C in a reaction mixture (final volume 25 μl) containing 20 mM Tris/HCl, pH 7.5, 8 mM $MgCl_2$, 20 μM ATP, 18.5kBq of [γ^{32}P]ATP, phosphate acceptors, and modifiers as indicated in each experiment. At this time point kinase activity was linear with the incubation time. Reactions were started by addition of enzyme fraction and terminated by spotting onto a Whatman P81 paper and submersion in 75 mM phosphate, and followed by three times of 10 min washes. Incorporation of ^{32}P phosphate into phosphate acceptors was assessed by scintillation counting.

Preparation of antisera: Antiserum against N-terminal region or C-terminal region of PKN was obtained by immunizing rabbits with the bacterial synthesized fragments of amino acids 1-391 for N-terminal region, and 637-946 for C-terminal region of rat PKN, respectively.

Immunoblotting: Samples were separated by SDS-PAGE, and the proteins were transferred to a PVDF membrane (Millipore). The membrane was incubated with each antiserum, and the immunoreactive bands were visualized by Enhanced Chemiluminescence (ECL) Western blotting detection kit (Amersham Corp.).

Immunoprecipitation: A 100-μl aliquot of Resource Q fraction #4 was incubated with 2 μl of antiserum against N-terminal region of PKN for 2 hr in 400 μl of buffer B (20 mM Tris/HCl, pH 7.5 containing 1 M NaCl, 1 % NP40, 1 mM PMSF, 0.1 mM Na vanadate, 10 mM NaF, and 1 μg/ml leupeptin), then 150 μl of 20 % protein A Sepharose was added. After 1h incubation at 4°C, the immunoprecipitates adsorbed to protein A Sepharose were washed three times with buffer C (10 mM Tris/HCl, pH 7.5 containing 0.5 M NaCl, 0.5 % NP40, 0.5 mM PMSF, 0.05 mM Na vanadate, 5 mM NaF, and 0.5 μg/ml leupeptin) and twice with buffer D (20 mM Tris/HCl, pH 7.5 containing 1 mM EDTA and 1 mM EGTA). The resulting immunoprecipitates were mixed in a final volume of 80 μl of the same buffer and 10-μl aliquot was subjected to the kinase assay using εPKC peptide as a phosphate acceptor as described above. Another 10-μl aliquot was subjected to kinase assay without exogenous substrate, then washed once with buffer C and subjected to 10 % SDS-PAGE followed by autoradiography.

Limited tryptic digestion: A 15-μl aliquot of Resource Q fraction #4 were trypsinized in a total volume of 30 μl containing 10 mM Tris/HCl, pH 7.5, 0.2 M β-mercaptoethanol, and 25 μg/ml trypsin. Incubations were for the indicated time in figures at 32°C, and were stopped by addition of 2 μl of 20 mg/ml soybean trypsin inhibitor and cooled on ice. Lipid-dependent and lipid-independent kinase activity were then assessed by testing 5 μl of the reaction mixture, and subjected to the kinase assay as described above. Another 10-μl aliquot was subjected to 12.5 % SDS-PAGE followed by immunoblotting using antiserum against C-terminal region of PKN.

RESULTS

Partial purification of PKN and effects of lipids on PKN kinase activity: PKN was purified partially from the soluble cytosolic fraction of COS7 cells transfected with human PKN expression construct, by ammonium sulfate fractionation and successive Resource Q column chromatography which was connected to FPLC system. As shown in Fig. 1B, assay of eluate from Resource Q column using εPKC peptide as a substrate revealed a broad peak of kinase activity. Kinase activity of PKN in these fractions, measured using subtraction of phosphate incorporation of εPKC peptide by control construct-transfected COS7 cells from by PKN cDNA construct-transfected cells, was relatively low for its protein content estimated by 120-kDa PKN immunoreactivity (Fig. 1 C). We speculate that PKN activity might be regulated by the co-factors, and examined a number of candidate modifiers of PKN kinase activity (Table I). The fraction with peak of PKN immunoreactivity (Fraction #4) from PKN cDNA construct-transfected COS7 cells and the corresponding fraction from control construct-transfected cells were used for the estimation of PKN kinase activity in the following experiment.

As shown in Table I, neither cyclic nucleotide including cAMP and cGMP, or ssDNA enhanced kinase activity of PKN significantly (Table I), although N-terminal region of PKN contains α–helical leucine zipper-like sequences and adjacent basic region, suggesting its ability to associate DNA (1, 4, 5). Calmodulin is capable of recognizing basic amphiphilic α-helical structure independent of the precise details of their amino acid sequences and regulate various cellular components (6), but kinase activity of PKN was not enhanced by either calmodulin alone or Ca^{2+}/calmodulin. Then we further examined whether Ca^{2+}/lipids modified kinase activity of PKN. As shown in Table I, unsaturated fatty acids including arachidonic acid, linoleic acid, and oleic acid activated PKN potently, whereas either Ca^{2+}/PS/diolein which was potent activator of PKC (7), other phospholipid tested, or saturated fatty acid such as palmitic acid were less effective.

In the presence of 40 μM arachidonic acid, assay of all fractions of eluate from Resource Q column revealed a single major peak of protein kinase activity (Fig. 1B). The activity of this peak fraction eluted at about 0.35M NaCl from the PKN cDNA construct-transfected COS7 cells was approximately three times greater than that from the control construct-transfected cells (Fig. 1A and

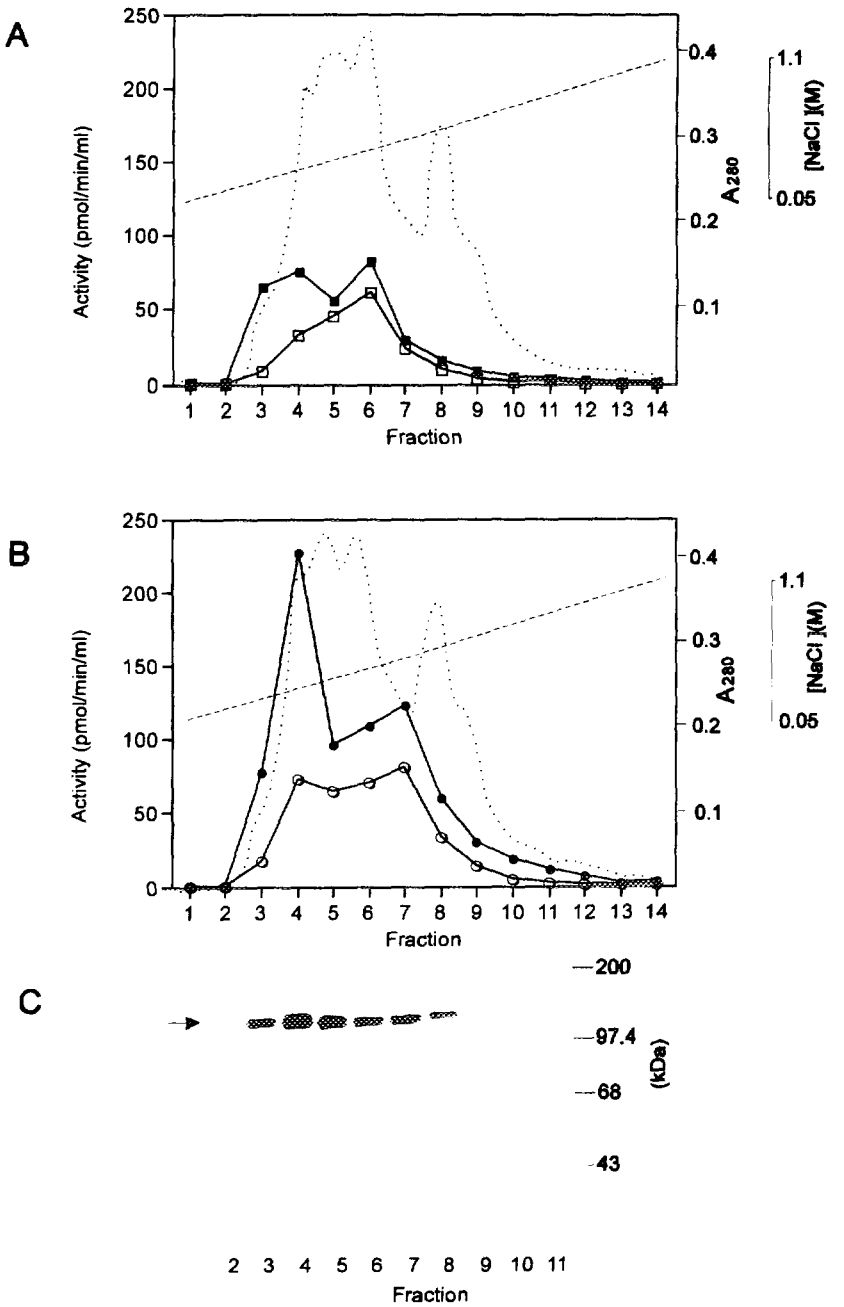


Fig. 1. Resource Q column chromatography of PKN. A 20-40% ammonium sulfate fraction, prepared from COS7 cells transfected with control construct (A) or PKN cDNA construct (B), was purified further by Resource Q column separation. 5-μl aliquots of fractions were assayed for arachidonic acid-independent (open circles and squares) or arachidonic acid-dependent (filled circles and squares) εPKC peptide kinase activity (-----), NaCl concentration (·····), absorbance at 280nm. Ten-μl aliquots of fraction 1-11 were subjected to 10 % SDS-PAGE and immunoblotting (C) using antiserum against N-terminal region of PKN. Positions of the molecular masses (kDa) are indicated on the right.

Table I
Effects of various modifiers on PKN activity

Modifier (s)	% of Control Activity
cAMP (10 μM)	115
cGMP (10 μM)	102
ssDNA (50 μg/ml)	137
Calmodulin (1 mg/ml)	95
Calmodulin (1 mg/ml) +Ca^{2+}(1 mM)	13
PS (8 μg/ml)+ diolein (0.8 μg/ml) +Ca^{2+} (1 mM)	94
PC (8 μg/ml)	70
Lyso PC (8 μg/ml)	59
PE (8 μg/ml)	104
Lyso PE (8 μg/ml)	110
Arachidonic acid (40 μM)	770
Linoleic acid (40 μM)	818
Palmitic acid (40 μM)	153
Oleic acid (40 μM)	641

Fraction #4 from Resource Q column chromatography described in Fig. 1 was assayed for εPKC peptide kinase activity in the presence of various modifiers as described in "MATERIALS AND METHODS". Phosphate incorporation by fraction #4 of control construct-transfected COS7 cells tested under each modifier condition was subtracted from that by fraction #4 of PKN cDNA construct-transfected cells. Phosphate incorporation in the absence of modifiers was 20 pmol/min/ml of fraction, which was taken as 100% activity. These data were representative of three independent experiments.

1B). Western blot analysis of these fractions with antiserum made against N-terminal region of PKN revealed a major peak of 120-kDa immunoreactivity consistent with that of kinase activity dependent of arachidonic acid (Fig. 1B and 1C).

Substrate specificity: C-terminal catalytic domain of PKN is highly homologous to the corresponding region of PKCs (1). Therefore a number of substrates, especially preferable for PKCs, have been used to examine PKN kinase activity. In the absence of modifiers, Histone H1, protamine sulfate, and MBP were relatively efficient substrate tested (Table II). Forty μM arachidonic acid enhanced kinase activities of PKN for these substrates only about 2-fold, whereas increased activities for synthetic peptides based on the PKC pseudosubstrate sites or ribosomal S6 protein about 6~50-fold over control (-arachidonic acid) activity.

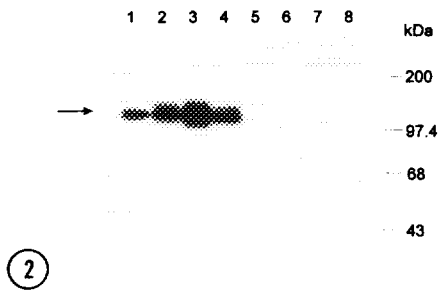
Table II
Substrate specificity of PKN

Substrate	Kinase Activity (pmol/min/ml)	
	-Arachidonic acid	+Arachidonic acid (40 μM)
Histone H1 (0.2 mg/ml)	24	50
Whole histone (0.2 mg/ml)	10	21
Protamine sulfate (0.2 mg/ml)	91	123
MBP (0.2 mg/ml)	21	36
Casein (0.2 mg/ml)	5	9
αPKC peptide (40 μM)	3	130
δPKC peptide (40 μM)	3	119
εPKC peptide (40 μM)	10	104
ζPKC peptide (40 μM)	5	37
S6 peptide (40 μM)	8	90

Fraction #4 from Resource Q column chromatography described in Fig. 1 was assayed under various substrate conditions. Kinase assays were as described in "MATERIALS AND METHODS". Phosphate incorporation by fraction #4 of control construct-transfected COS7 cells under each substrate condition was subtracted from that by fraction #4 of PKN cDNA construct-transfected cells. These data were representative of three independent experiments.

Immunoprecipitation of PKN: To further substantiate these observations, it was determined whether PKN retains its properties upon further purification. PKN was immunoprecipitated with antiserum against N-terminal region of PKN from fraction #4 of Resource Q column chromatography, and subjected to autophosphorylation assay. Autophosphorylation of the immunoprecipitates from PKN cDNA construct-transfected COS7 cells was enhanced by various fatty acids (Fig. 2), and the extent of phosphate incorporations were almost parallel to the εPKC peptide kinase activities of immunoprecipitates (data not shown). Autophosphorylation activity of endogenous PKN in COS7 cells was not detected in the absence of modifiers, while it was faintly detected in the presence of arachidonic acid (Fig. 2, lane 7).

Limited proteolysis of PKN: Pretreatment of trypsin induced enhancement of εPKC peptide kinase activity (from 25 pmol/min/ml to 110 pmol/min/ml; Fig. 3, open circles). Trypsin pretreatment of PKN decreased the amount of intact enzyme and induced the formation of proteolytic fragments of apparent molecular mass of 50 and 43 kDa that could be recognized by antiserum against C-terminal region of PKN (Fig. 3, *Inset*). The appearance of εPKC peptide kinase activity was most closely correlated with the appearance of the 43-kDa fragment. In order to generate a fragment of that size, the cleavage site must lie near residue 600 if the C-terminal end of PKN was preserved from tryptic



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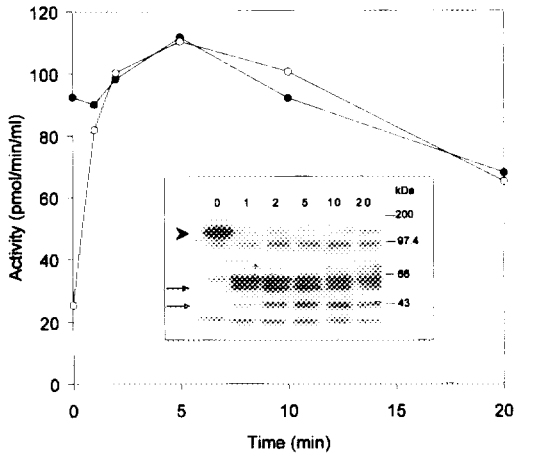

(3)

Fig. 2. Immunoprecipitation and autophosphorylation of PKN.
Immunoprecipitates from PKN cDNA construct (lanes 1-4)- and control construct (lanes 5-8)-transfected COS7 cells were autophosphorylated with palmitic acid (lanes 1 and 5), oleic acid (lanes 2 and 6), arachidonic acid (lanes 3 and 7), and without lipid (lanes 4 and 8) as described in "MATERIALS AND METHODS". Arrow indicates the position of autophosphorylated PKN. Positions of the molecular masses (kDa) are indicated on the right.

Fig. 3. Limited proteolysis of PKN. Fraction #4 from Resource Q column chromatography described in Fig. 1 was pretreated with 25 μg/ml trypsin as indicated time. Five μl digested samples were assayed for εPKC peptide kinase activity in the absence (open circles) or presence (closed circles) of arachidonic acid as described in "MATERIALS AND METHODS". Background was determined in an identical experiment but using a fraction prepared from control construct-transfected COS7 cells and was subtracted for each condition. Means of duplicate incubations are shown. *Inset*, Western blotting of Resource Q column chromatography fractions from PKN cDNA-construct-transfected COS7 cells after pretreatment with trypsin. The polyclonal antiserum against C-terminal region of PKN was used for immune detection. Several nonspecific bands were detected with this antiserum as they were also present in the case of control construct-transfected cells (data not shown). 0, 1, 2, 5, 10, and 20 indicate pretreatment with 0, 1, 2, 5, 10, 20 min with 25 μg/ml trypsin,respectively. The arrowhead indicates intact PKN. The arrows (open and closed) indicate PKN tryptic fragments. Positions of the molecular masses (kDa) are indicated on the right.

cleavage. Cleavage in this region would mean that the 43-kDa fragment contained the catalytic domain of PKN. This indicates that N-terminal region of PKN restrict εPKC peptide kinase activity of intact PKN. εPKC peptide phosphorylation by PKN pretreated for more than 2 min with trypsin was the same extent as that in the presence of 40 μM arachidonic acid, showing that this proteolytic form of PKN was 40 μM arachidonic acid-independent.

DISCUSSION

To characterize the PKN protein, we reported the expression of PKN cDNA using a baculovirus system, and revealed that almost all recombinant PKN was recovered in the Triton/salt-insoluble fraction by biochemical fractionations (1). In PKN cDNA construct-transfected COS7 cells, PKN was also recovered in the Triton/salt-insoluble fraction. However, immunocytochemical experiment using PKN cDNA construct-transfected COS7 cells revealed PKN immunoreactive

staining was rather not a nuclear but a perinuclear or cytoplasmic pattern (data not shown), and biochemical fractionation of native rat tissues (brain, lung, spleen, and testis) indicated that the enzyme was rather mainly localized in the soluble cytosol (manuscript in preparation). These data suggest that high-level expression of PKN in insect cells or COS7 cells might produce many incorrectly folded molecules. Relatively low specific kinase activity of Triton/salt-insoluble PKN using our assay conditions (assayed in resuspended state of the enzyme, data not shown) might in part originate from the pelletable PKN structure. To understand the enzymology and cellular mechanisms involved in regulating PKN activity, in this paper we used the soluble cytosolic fraction of PKN cDNA construct-transfected COS7 cells as the enzyme source.

PKN was isolated by cDNA cloning on the basis of nucleotide sequence similarity to the catalytic domain of PKC, but its enzymatic properties were distinct from that of Ca^{2+}-dependent isoforms of PKC since its activity was not dependent or rather suppressed by Ca^{2+}. Moreover properties of this enzyme was distinct from that of Ca^{2+}-independent isozyme of PKC (3, 8) since it lacks responsiveness to PS/diolein. Therefore PKN does not belong to PKC family in view of biochemical properties, it may be a member of an extended family of lipid-dependent protein kinases since this enzyme was dependent on unsaturated fatty acids for activation. Recently Wasiuddin et al. reported the partial purification of *cis*-unsaturated fatty acid activated kinase from human platelets, which was distinct from known isozymes of PKC (9). Whether it is PKN or other member of lipid-dependent kinase family must await sequence analysis.

Activation of PKN by limited proteolysis and unresponsiveness of proteolytic PKN to arachidonic acid suggest that unsaturated fatty acids were capable to remove restriction of catalytic activity by N-terminal region of the enzyme. Primary structure of N-terminal half of PKN shows no significant similarity to PKCs (1), and lacks typical pseudosubstrate region involved in the intramolecular inhibition of the enzyme (10) as observed in PKCs. Elucidation of the role of N-terminal region of PKN to restrict kinase activity may provide the information for the cellular signal pathway through PKN.

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