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ORIGINAL PAPER

Depletion of the catalytic subunit of protein phosphatase-2A (PP2Ac) markedly attenuates glucose-stimulated insulin secretion in pancreatic β-cells

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Abstract Among various phosphatases, the protein phosphatase 2A (PP2A) is relatively well studied in the islet. Previously, we have demonstrated that the catalytic subunit of PP2A (PP2Ac) undergoes okadaic acid (OKA)-sensitive, reversible carboxylmethylation (CML), which appears to be requisite for glucose-stimulated insulin secretion (GSIS). Using the siRNA approach, we examined, herein, the contributory roles of PP2Ac in GSIS from insulin-secreting pancreatic β-(INS-1 832/13) cells. Immunologically, PP2Ac was detectable in all the subcellular fractions studied in rank order of: cytosol > microsomes > secretory granules = nucleus > mitochondria. Transfection of PP2Ac-specific, but not scrambled-siRNA, markedly attenuated PP2A activity and GSIS in these cells. Together, our findings provide a direct evidence for a positive modulatory role for PP2Ac in signaling steps leading to GSIS.

Keywords Pancreatic β-cells · INS-1 832/13 cells · Protein phosphatase 2A · Glucose-stimulated insulin secretion

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Introduction

It is widely accepted that glucose-stimulated insulin secretion (GSIS) from pancreatic β-cells involves the generation and/or alterations in the intracellular levels of second messengers, such as cyclic nucleotides, ions and lipid hydrolytic products of phospholipase A_2, D, or C [1]. Some of the known actions of these modulators include, but are not limited to, regulation of a cascade of protein kinases endogenous to the pancreatic β-cells. Indeed, several earlier studies have demonstrated the localization of such kinases in normal rat islets as well as clonal β-cells. They include calcium-, calcium–calmodulin-, cAMP-, and phospholipid-dependent protein kinases [2]. Using selective inhibitors of these kinases, it has been possible to demonstrate regulatory roles for endogenous protein phosphorylation in insulin exocytosis [2, 3].

The phosphorylation status of proteins is regulated by the balance of the activities of protein kinases and phosphatases, which induce the addition and removal of phosphate from these proteins, respectively. Although several earlier studies have focused on the identification and characterization of protein kinases, relatively very little information is available on the localization of protein phosphatases in β-cells. In this context, some of the previous studies have suggested an important role for protein phosphatase 2A (PP2A) in GSIS [3–8]. The PP2A family of enzymes represents a major class of serine/threonine protein phosphatases, which have been implicated in the regulation of many cellular events [9]. Several holoenzyme complexes have been isolated and characterized from a variety of tissues [3, 9].

Typically, the PP2A heterodimer complex is comprised of a scaffolding A subunit with an apparent molecular-mass of 65 kDa and the 36 kDa catalytic subunit, PP2Ac. This A/C heterodimer interacts with the regulatory B

subunit yielding the PP2A holoenzyme. The binding of regulatory B subunit to the A/C heterodimer is felt to provide further stability to the holoenzyme [9]. It is also suggested that the variable B subunit(s) influences substrate specificity and/or subcellular localization of a given PP2A holoenzyme complex [10]. It is estimated that the combination of different subunits (e.g., A, B, and C) could produce >75 different trimeric holoenzymes, although the precise number of the possible holoenzyme complexes that actually exist in cells still needs to be determined [9, 11]. While the A and C subunits are ubiquitously expressed, certain B subunits are expressed in a tissue-specific manner and at various stages of cellular development [9, 11].

Recent years have witnessed significant progress in the area of functional regulation of PP2A, specifically via post-translational modification (e.g., carboxylmethylation; CML) steps [5, 6, 12–16]. Indeed, we have provided the first evidence that the CML of PP2Ac plays a major regulatory role in islet function [5, 6, 8]. We have been able to demonstrate that OKA, but not its inactive analog, norokadaone, specifically inhibit the CML and associated phosphatase activity in a variety of insulin-secreting cells, including normal rat islets, HIT-T15 cells, and INS-1 cells [5]. In the context of localization of PP2A in the islet β-cell, we previously characterized a cytosolic PP2A, which is activated by glutamate and magnesium (referred to as glutamate and magnesium-activated protein phosphatase; GAPP), which catalyzes the dephosphorylation and activation of acetyl-CoA carboxylase, and subsequent generation of long-chain fatty acyl derivatives, which are necessary for GSIS [17]. Together, these data suggest a positive modulatory role for a specific form of PP2A in GSIS. In the current studies, we investigated potential regulatory roles for PP2Ac in GSIS from insulin-secreting INS-1 832/13 cells by selectively depleting the endogenous expression of PP2Ac via the RNAi approach. Our findings provide the first evidence to suggest that PP2Ac plays key regulatory functions in the signaling steps leading to GSIS.

Materials and methods

Materials

Okadaic acid was purchased from Biomol Research Laboratories (Plymouth Meeting, PA). The serine/threonine phosphatase assay kit and PP2Ac siRNA/siAb^{TM} assay kit were purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Affinity purified, polyclonal antiserum directed against PP2Ac and PP2A/Aα were from Santa Cruz Biotechnology (Santa Cruz, CA). The rat insulin ELISA kit was from American Laboratory Products (Windham, NH). Rac1 antibody was obtained from BD Bioscience (San Jose, CA). All other reagents used were of highest purity available.

Islet isolation

Pancreatic islets from male Sprague-Dawley rats (200–250 g body wt; Harlan Laboratories) were isolated by collagenase digestion method as we described in [18, 19].

Insulin-secreting INS-1 832/13 cells

INS-1 832/13 cells (kindly provided by Dr. Chris Newgard, Duke University Medical Center, Durham, NC) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). The medium was changed twice weekly and cells were trypsinized and subcloned weekly.

Subcellular distribution of PP2Ac in INS-1 832/13 cells and rat islets

Islets or INS-1 832/13 cells were homogenized in the buffer consisting of 230 mM mannitol, 70 mM sucrose, and 5 mM HEPES, pH 7.4, containing 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail. For subcellular fractionation, the homogenates were centrifuged at 900*g* to remove the nuclei and unbroken cells. The post-nuclear supernatant was then centrifuged at 5,500*g* for 15 min, 25,000*g* for 20 min, and 105,000*g* for 60 min, to obtain mitochondria, secretory-granule, and microsomal enriched fractions respectively, in a Beckman Optima TL ultracentrifuge. The purity of each of these subcellular fractions has been previously determined in our laboratory [20]. The proteins from each of these fractions were separated by SDS-PAGE, and the resolved proteins were transferred to nitrocellulose membrane. The membranes were then blocked and incubated with antibodies directed against PP2Ac or PP2A/Aα followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody. Immune complexes were detected using enhanced chemiluminescence (ECL) detection kit.

siRNA-mediated knockdown of PP2Ac in INS-1 832/13 cells

The endogenous PP2Ac expression was inhibited by transfecting cells using small interfering RNA (siRNA). A 21-

oligonucleotide RNA forming a 19 bp duplex core with two nucleotide "UU" 3′-overhangs and a 5′ phosphate on the antisense strand or scrambled-siRNA (negative control) were obtained from Upstate Cell Signaling (Lake Placid, NY). Cells were transfected with PP2Ac-siRNA at a final concentration of 100 nM using HiPerfect transfection reagent obtained from Qiagen (Valencia, CA). To assess specificity of RNAi method, cells were transfected (as above) with non-targeting RNA this includes at least four nucleotide mismatches with all known mouse, rat, and human genes (i.e., scrambled-siRNA) duplexes (also obtained from Upstate Cell Signaling). Transfected cells were maintained in complete growth medium for 48 h. The efficiency of PP2Ac knockdown was determined by Western blot analysis and catalytic activity of PP2A (see Results).

Glucose-stimulated insulin secretion

Control or PP2Ac-depleted β-cells were cultured overnight in low glucose containing media. They were further incubated in the presence of either low (5 mM) or high (20 mM) glucose for 30 min at 37°C in the continuous absence or presence of inhibitors as indicated in the text. Insulin released into the medium was quantitated by ELISA [21].

Protein assay

Protein concentration in the lysates and subcellular fractions from islets and INS-1 832/13 cells was determined by dye-binding method of Bradford using BSA as the standard [22].

Statistical analyses of experimental data

Statistical significance of differences was determined by ANOVA. A P-value <0.05 was considered significant.

Results

Subcellular distribution of PP2Ac in insulin-secreting cells

At the outset, we determined, by Western blotting, localization of PP2Ac in INS-1 832/13 and normal rat islet lysates. Data in Fig. 1 (panel A) verify the presence of this protein in each of the two cellular preparations. We next determined the localization of PP2Ac and PP2A/Aα (the structural/scaffolding subunit of the holoenzyme) in

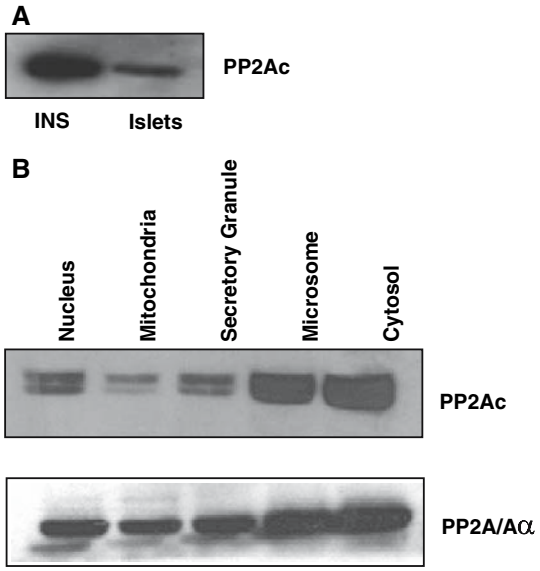

Fig. 1 *Subcellular distribution of PP2Ac in INS-1 832/13 and normal rat islets:* (Panel **A**) INS-1 832/13 or islet protein lysates (40 μg) were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane. The membranes were then blocked and incubated with anti-PP2Ac serum (1:1,000 dilutions; 1 h) followed by incubation with HRP conjugated secondary antibody (1:1,000 dilution) for 1 h. Immune complexes were detected using enhanced chemiluminescence (ECL) kit. A representative blot from three experiments yielding similar results is shown here. (Panel **B**) Subcellular fractions were isolated from INS-1 832/13 cells by differential centrifugation (see Methods). Proteins (40 μg) from these fractions were separated by SDS-PAGE followed by transfer to a nitrocellulose membrane. The membranes were then blocked and incubated with either anti-PP2Ac serum (upper panel; 1:1,000 dilutions; 1 h) or with antibody raised against PP2A/Aα (lower panel; 1:1,000 dilutions; 1 h) followed by incubation with HRP conjugated secondary antibody. Immune complexes were detected using ECL kit. A representative blot from three experiments yielding similar results is shown here

different subcellular compartments isolated from INS-1 832/13 cells (see Methods). Data in Fig. 1 (panel B) demonstrate that the PP2Ac (upper panel) is localized in all subcellular fractions studied, albeit with varying degrees of abundance. The relative degree of abundance for PP2Ac in INS-1 832/13 cells is in the following rank order: cytosol > microsomes > secretory granules = nucleus > mitochondria. In a manner akin to PP2Ac, the PP2A/Aα subunit is localized in all subcellular fractions examined (Fig. 1; panel B). We observed relatively higher degree of abundance of this subunit in the cytosolic and microsomal fractions compared to secretory granule, nucleus, or mitochondria (Fig. 1; panel B).

Depletion of endogenous PP2Ac markedly reduces PP2A activity in pancreatic β-cells

As stated above, previous studies from our laboratory have reported that PP2Ac undergoes post-translational CML in

normal rat islets and clonal β-cell preparations [5]. However, potential regulatory roles for this protein in GSIS remain unknown. With this in mind, we quantitated GSIS in INS-1 832/13 cells in which the endogenous expression of PP2Ac was specifically knocked down via the siRNA approach (see Methods). Data in Fig. 2 (panel A) indicate >50% reduction in the expression of PP2Ac under our current experimental conditions. Such a reduction in PP2Ac expression culminated in >60% reduction in the OKA-sensitive PP2A activity in cognate cellular preparations (1.051 ± 0.16 pmoles of p-NPP hydrolyzed/min/μg protein in control cell lysates versus 0.369 ± 0.10 pmoles of p-NPP hydrolyzed/min/μg protein, in cells in which PP2Ac was knocked down; $n = 3$ determinations in each case). Together, these data indicate that specific knockdown of the PP2Ac results in reduction in the expression as well as PP2A enzymatic activity in insulin-secreting cells.

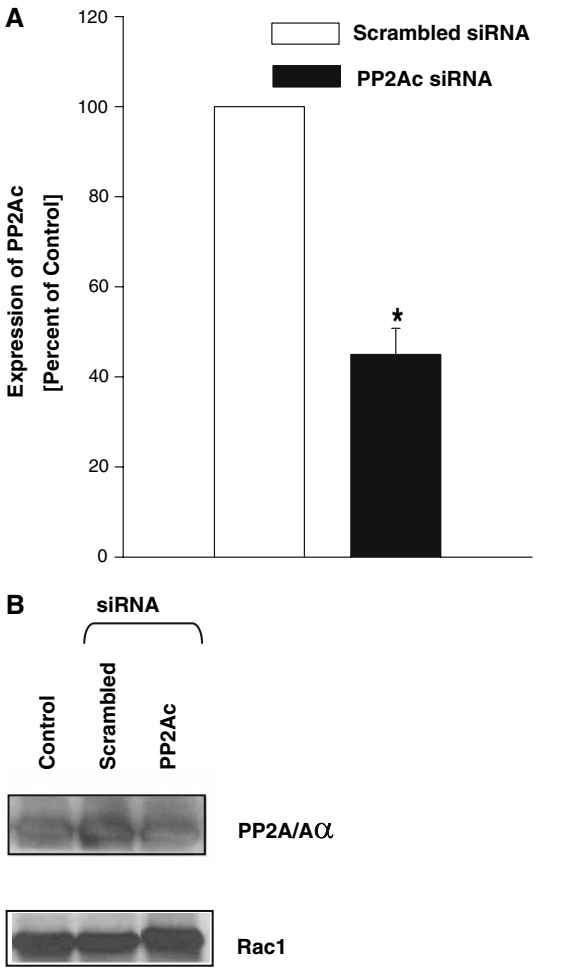

Fig. 2 *Expression levels of PP2Ac and PP2A/Aα and Rac1 in INS-1 832/13 cells after PP2Ac knockdown by RNAi:* (Panel **A**) INS-1 832/13 cells were transfected with PP2Ac-siRNA or scrambled-siRNA at a final concentration of 100 nM using HiPerfect Transfection reagent (Valencia, CA). Lysates (30 μg protein) from cells transfected with scrambled-siRNA or PP2Ac-specific siRNA were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with an antibody raised against PP2Ac. Immune complexes were detected using ECL kit. Relative intensities of the protein bands were quantitated by densitometry. Data, which are represented as percent of scrambled-siRNA, are mean ± SEM from 3 different experiments. *Represents $P < 0.05$. (Panel **B**) Lysates (20 μg protein) from non-transfected cells, cells transfected with scrambled-siRNA or PP2Ac-specific siRNA were separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with antibodies raised against PP2A/Aα (1:1,000 dilutions; 1 h) or Rac1 (1:1,000 dilutions; 1 h). Immune complexes were detected using ECL kit. A representative blot from three experiments yielding similar results is shown here

To rule out the possibility that the observed reduction in PP2A activity in PP2Ac silenced cells is due to non-specific knockdown of other PP2A subunits, we verified potential alterations, if any, in the expression of the structural subunit of PP2A (PP2A/Aα) in lysates derived from control or PP2Ac-depleted cells. Data in Fig. 2 (panel B) suggest no significant differences in the expression of PP2A/Aα between the control, scrambled, and PP2Ac silenced cells. Further, we also determined relative abundance of Rac1, a small G-protein, which has been implicated in GSIS [19, 23] in control cells and in cells in which PP2Ac expression was compromised via the siRNA method. Data in Fig. 2 (panel B) also suggest that our experimental manipulation to silence the expression of PP2Ac elicited no effect on the expression levels of Rac1. Furthermore, we were unable to detect any significant differences in the expression of tubulin in cells transfected with PP2Ac-siRNA compared to control and scrambled-siRNA transfected cells (data not shown). Taken together, these data indicate that our experimental approach to silence PP2Ac expression is specific for the protein in question.

Depletion of endogenous PP2Ac markedly reduces GSIS in pancreatic β-cells

We next quantitated GSIS in INS-1 832/13 cells in which PP2Ac levels are either retained at normal levels or compromised via the RNAi method. We also utilized a scrambled-siRNA preparation, as a negative control, to account for any potential non-specific effects of transfection procedure on GSIS. Data in Fig. 3 demonstrate a robust increase (3.5–4 fold) in GSIS in control cells challenged with a stimulatory concentration of glucose (20 mM; first bar). A comparable degree of stimulation of GSIS (3 fold) is also seen in cells expressing the scrambled-siRNA (bar 2). However, we noticed a marked inhibition in GSIS in cells in which endogenous expression of PP2Ac is reduced (bar 3). It may be germane to point out that knockdown of PP2Ac in these cells caused no significant increase in basal insulin secretion (data not shown). These findings indicate that PP2Ac plays a positive modulatory role in GSIS (see below).

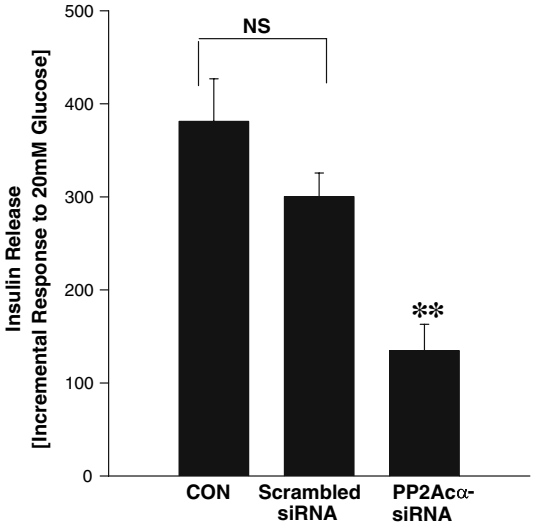

Fig. 3 *RNAi-mediated knockdown of PP2Ac markedly inhibits GSIS in INS-1 832/13 cells:* INS-1 832/13 cells were transfected with PP2Ac-siRNA or scrambled-siRNA at a final concentration of 100 nM using HiPerfect Transfection reagent. GSIS was quantitated in these cells as described in Methods. Data, which are expressed as percent basal, are means ± SEM from three experiments. *Represents $P < 0.01$ versus control and **Represents $P < 0.05$ versus non-transfected or scrambled-siRNA transfected cells

Discussion

The overall objective of this current study was to investigate the contributory roles for PP2Ac in GSIS in pancreatic β-cells. Our findings suggest that: (i) PP2Ac is localized in all the subcellular fractions, including insulin-containing secretory granules, albeit with a varying degree of abundance; (ii) siRNA-mediated knockdown of PP2Ac significantly reduces PP2A activity; and (iii) siRNA-mediated knockdown of PP2Ac markedly attenuates GSIS in these cells. Together, we believe that these findings establish a key regulatory role(s) for PP2Ac in the β-cell function.

Our current working model was based on two key observations to indicate PP2Ac might play regulatory roles in islet β-cell function. First, using isotope-labeling methods and vapor-phase equilibration assay, we have demonstrated that post-translational CML of PP2Ac is necessary for insulin secretion [5, 8]. Second, several earlier studies have utilized specific inhibitors of PP2A, such as OKA to examine its role in insulin-secretion [24–29], and indeed, we have also demonstrated that OKA specifically inhibits the CML of PP2Ac [5, 8]. Together, these findings implicate a principal role for PP2Ac in islet function. With these observations in mind, we undertook the current investigation to conclusively demonstrate that PP2Ac contributes toward GSIS in insulin-secreting INS-1 832/13 cells. Our findings provide the first direct evidence to suggest marked inhibition in GSIS in cells in which the endogenous expression and subsequent catalytic activation of an OKA-sensitive PP2A is compromised via gene silencing methodology.

A perusal of the existing body of evidence clearly implicates stimulatory as well as inhibitory roles for OKA-sensitive protein phosphatases in GSIS from the pancreatic β-cell. For example, Ratcliff and Jones [27] demonstrated stimulatory effects of OKA, a selective blocker of protein phosphatases, on basal and cAMP-induced insulin secretion from pancreatic islets. Murphy and Jones [29] have demonstrated inhibition of GSIS in normal rat islets following inhibition of endogenous PP1/PP2A activities. They also provided evidence to suggest stimulation of PP1/PP2A activities by glucose in these cells. Sato et al. [25] observed significant inhibition, by OKA, of GSIS in mouse islets. These and several other related studies [2, 3, 25, 27, 29, 30] appear to suggest that both phosphorylation and dephosphorylation of specific islet proteins are necessary for GSIS. Furthermore, the subject of involvement of PP2A in islet function becomes much more complex when one studies the requirement of phosphorylation–dephosphorylation signaling steps for insulin secretion elicited by insulin secretagogues, other than glucose. This is especially true in light of the multifactorial regulation of PP2A by various modulators, such as amino acids, ions, purine nucleotides, polyamines, sulfonylureas, cyclic nucleotides, inositol phosphates, cytokines, ceramides, etc. (3 for a review). Therefore, it is likely that different signaling steps could govern and control insulin exocytosis, and, in each case, respective molecular and cellular mechanisms may be differentially affected by endogenous protein phosphorylation–dephosphorylation in the pancreatic islet β-cell [3].

It may be germane to point out that PP2Ac exists in α and β isoforms, which are closely related. Both of these isoforms comprise of 309 amino acids and share 97% sequence homology [9, 31]. Further, earlier studies have demonstrated that the degree of expression of the PP2Acα is at least 10 times greater than the PP2Acβ isoform, which is felt due to differences in transcriptional regulation [32]. Moreover, recent studies by Ikehara and co-workers [33] have clearly suggested remarkable similarities between the two isoforms in terms of their ability to: (i) hydrolyze p-NPP and phosphopeptide substrates; (ii) undergo inhibition by OKA and microcystin LR; (iii) associate with A and B subunits of the PP2A holoenzyme; and (iv) undergo methylation at their C-terminal leucyl residues. It is for these reasons, we feel it would be extremely difficult to pin point the precise roles of each of these isoforms in GSIS. It must be noted that to further address this issue, we used RNAi methodology to decipher roles for PP2Ac in islet function and in GSIS. While our current data provide convincing evidence in support of a regulatory role for

PP2Ac in GSIS, additional studies are clearly needed to further identify the candidate phosphoproteins, whose dephosphorylation might trigger their functional activation. These may include, but not limited to, the AMP-kinase and acetyl-CoA carboxylase signaling cascade, since each of these proteins is known to require phosphorylation/dephosphorylation steps for their functional activation [17].

In conclusion, we feel that a better understanding of the identity of the individual subunits of the PP2A holoenzyme (i.e., the A, B, and C subunits) is necessary to further decipher the modes of action of individual insulin secretagogues, which might exert direct effects on the PP2A function either by regulating the post-translational modifications of the subunits (e.g., CML) or by directly affecting the holoenzyme assembly. To this end, at least in the context of GSIS, we provide the first direct evidence to indicate that PP2Ac plays a positive modulatory role(s) in GSIS from INS-1 832/13 cells. Future studies will need to conclusively identify the A and B subunits of the PP2A, as well as the locus (or loci) of action PP2A which regulate GSIS. Lastly, we believe that our current demonstration of importance of PP2Ac should provide basis for future work to understand the nature of and the mechanisms underlying the defects in OKA-sensitive phosphatase signaling steps that we recently reported in islets from the Goto-Kakizaki rat, a model for spontaneous type 2 diabetes [18].

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