Control of Phosphatidylserine Synthase II Activity in Chinese Hamster Ovary Cells*

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Phosphatidylserine (PtdSer) in Chinese hamster ovary (CHO) cells is synthesized through the action of PtdSer synthase (PSS) I and II, which catalyzes the exchange of L-serine with the base moiety of phosphatidylcholine and phosphatidylethanolamine, respectively. The PtdSer synthesis in a CHO cell mutant, PSA-3, which lacks PSS I but has normal PSS II activity, was almost completely inhibited by the addition of PtdSer to the culture medium, like that in the wild-type CHO-K1 cells. In contrast, the PtdSer synthesis in a PSS II-overproducing stable transformant of CHO-K1, K1/wt-pssB, was reduced by only 35% upon addition of PtdSer. The serine exchange activity in a membrane fraction of K1/wt-pssB cells was not inhibited by PtdSer at all, whereas those of PSA-3 and CHO-K1 cells were inhibited by >95%. These results indicated that PSS II activity in PSA-3 and CHO-K1 cells is inhibited by exogenous PtdSer and that overproduction of PSS II leads to the loss of normal control of PSS II activity by exogenous PtdSer. Although overproduced PSS II in K1/wt-pssB cells was not normally controlled by exogenous PtdSer, K1/wt-pssB cells cultivated without exogenous PtdSer exhibited a normal PtdSer biosynthetic rate similar to that in CHO-K1 cells. In contrast to K1/wt-pssB cells, another stable transformant of CHO-K1, K1/R97K-pssB, which overproduces R97K mutant PSS II, exhibited a ~4-fold higher PtdSer biosynthetic rate compared with that in CHO-K1 cells. These results suggested that for maintenance of a normal PtdSer biosynthetic rate, the activity of overproduced wild-type PSS II in K1/wt-pssB cells is depressed by an as yet unknown post-translational mechanism other than those for the exogenous PtdSer-mediated inhibition and that Arg-97 of PSS II is critical for this depression of overproduced PSS II activity. When the cDNA-directed wild-type and R97K mutant PSS II activities were expressed at nonoverproduction levels in a PSS I- and PSS II-defective mutant of CHO-K1 cells, expression of the mutant PSS II activity but not that of the wild-type PSS II activity induced the PtdSer-resistant PtdSer biosynthesis. This suggested that Arg-97 of PSS II is critical also for the exogenous PtdSer-mediated inhibition of PSS II.

Phosphatidylserine (PtdSer) is an essential phospholipid for the growth of mammalian cells (1, 2), comprising approximately 10% of the total membrane phospholipids of various mammalian tissues and cultured cells. PtdSer formation in mammalian cells occurs through the exchange of L-serine with the base moiety of phosphatidylcholine (PtdCho) or phosphatidylethanolamine (PtdEtn) (2–4). The serine base exchange in Chinese hamster ovary (CHO) cells is catalyzed by at least two different enzymes named PtdSer synthase (PSS) I and II (1–8), which are encoded by the pssA and pssB genes, respectively (6, 8). PSS I is responsible for the conversion of PtdCho to PtdSer (2–4), and PSS II is responsible for the conversion of PtdEtn to PtdSer (2).

The PtdSer biosynthesis in CHO-K1 cells is remarkably inhibited upon addition of PtdSer to the culture medium (9), suggesting that feedback control is involved in the regulation of PtdSer biosynthesis. Because the serine base exchange activities in homogenates of CHO-K1 cells grown with and without exogenous PtdSer are essentially the same (9), the cellular levels of PSS I and PSS II appear to remain unchanged upon addition of PtdSer. In addition, it has been shown that PtdSer inhibits the serine base exchange activity in a membrane fraction prepared from a homogenate of CHO-K1 cells (10). These observations imply that the inhibition of serine base exchange activity by PtdSer is involved in the regulation of PtdSer biosynthesis.

A CHO cell mutant, named 29, whose PtdSer biosynthesis is highly resistant to inhibition by exogenous PtdSer, has been isolated from CHO-K1 cells (10). In a medium without exogenous PtdSer, mutant 29 cells synthesize PtdSer at a 2–3-fold higher rate and exhibit a ~2-fold higher cellular PtdSer level compared with those in CHO-K1 cells (10). Recently, mutant 29 was shown to carry a missense mutation in the pssA gene, which results in the replacement of Arg-95 of the gene product, PSS I, by Lys (11). The introduction of the mutant pssA cDNA isolated from mutant 29 cells into CHO-K1 cells induces ~5-fold elevation of the PtdSer biosynthetic rate and ~2-fold elevation of the cellular PtdSer level upon cultivation in a medium without exogenous PtdSer, whereas the wild-type pssA cDNA is incapable of inducing such significant elevations (11). Furthermore, it has been shown that the R95K mutation in pssA renders the product, PSS I, resistant to the inhibition by exogenous PtdSer (11). Thus, Arg-95 of PSS I is a critical residue for the control of PSS I activity.

Although information on the control of the PSS I activity has increased as described above, little is known concerning the control of activity of PSS II encoded by the pssB gene. PSS I and PSS II are similar in sequence to each other: there is 38%
amino acid sequence identity between the two synthases (8). PSS II has an arginine residue at position 97, which corresponds to Arg-95 of PSS I identified as a critical residue for the control of PSS I activity. In this study, we constructed a mutant pssB cDNA, in which codon 97 was changed from Arg to Lys. Using the wild-type and resultant R97K mutant pssB cDNA clones, we obtained the data suggesting that the PSS II activity in CHO-K1 cells is controlled by at least two different post-translational mechanisms including PtdSer-mediated inhibition and that Arg-97 of PSS II is a critical residue for the control of PSS II activity.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—Strain CHO-K1 was obtained from the American Type Culture Collection. CHO-K1 cells and transformants of CHO-K1 cells constructed in this study were maintained in Ham’s F-12 medium supplemented with 10% newborn calf serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), and NaHCO₃ (1, 176 g/liter) under a 5% CO₂ atmosphere of 100% humidity at 37 °C. 

RESULTS

PtdSer Formation by Both PSS II and PSS I in CHO-K1 Cells Is Inhibited upon Addition of PtdSer to the Culture Medium—PSS II catalyzes the conversion of PtdEtn to PtdSer (2), whereas PSS I catalyzes the conversion of PtdCho to PtdSer (2-4). To determine whether the PtdSer formation through the action of both PSS II and PSS I is inhibited by exogenous PtdSer, we metabolically labeled CHO-K1 cells with each of PtdEtn (3), PtdCho (3–4), and PtdSer. To select transformants that exhibited serine base exchange activity similar to that of PSA-3 cells, the resultant G418-resistant transformants were subjected to the in situ colony assay for serine base exchange, and a transformant (designated as K1/wt-pssB) and a pSVR97K-pssB/neo-transformed clone (designated as K1/R97K-pssB) were purified by limited dilution.

Isolation of Stable pSVpssBneo and pSVR97K-pssB/neo Transfectants of CHO-K1 Cells—Each of pSVpssBneo and pSVR97K-pssB/neo was introduced into CHO-K1 cells by the calcium phosphate precipitation method (14), and G418-resistant transformants were selected in the growth medium containing 400 μg/ml of G418 (Life Technologies, Inc.). From the transformants, a pSVpssB/neo-transformed clone (designated as K1/wt-pssB) and a pSVR97K-pssB/neo-transformed clone (designated as K1/R97K-pssB) were purified by limited dilution.

Other Methods—Assaying of serine base exchange activity in cell homogenates was performed as described (1). Protein was measured according to Lowry et al. (15) using bovine serum albumin as a standard.

RESULTS

PtdSer Formation by Both PSS II and PSS I in CHO-K1 Cells

Isolation of Stable pSVpssBneo and pSVR97K-pssB/neo Transfectants of CHO-K1 Cells—Each of pSVpssBneo and pSVR97K-pssB/neo was introduced into CHO-K1 cells by the calcium phosphate precipitation method (14), and G418-resistant transformants were selected in the growth medium containing 400 μg/ml of G418 (Life Technologies, Inc.). From the transformants, a pSVpssB/neo-transformed clone (designated as K1/wt-pssB) and a pSVR97K-pssB/neo-transformed clone (designated as K1/R97K-pssB) were purified by limited dilution.

FIG. 1. Conversion of exogenous PtdEtn and PtdCho to PtdSer in CHO-K1 cells is inhibited by the addition of PtdSer to the culture medium. CHO-K1 cells were metabolically labeled with [32P]PtdEtn (A and B) or [32P]PtdCho (C and D) for 24 h at 37 °C in the growth medium supplemented with (hatched bars) or without (filled bars) 60 μM PtdSer, and then the radioactivities of cellular PtdSer (A and C), PtdEtn (B), and PtdCho (D) were determined.
CHO-K1 cells was strikingly reduced on cultivation with exogenous PtdSer, although there was no significant difference in the level of cellular [32P]PtdEtN and [32P]PtdCho between CHO-K1 cells cultivated with and without exogenous PtdSer. These results, together with our previous finding that the PtdSer formation in CHO-K1 cells is almost completely inhibited by exogenous PtdSer (11), indicated that PtdSer formation by both PSS II and PSS I in CHO-K1 cells is inhibited upon addition of PtdSer to the culture medium. For further confirmation that the PtdSer formation by PSS II is inhibited by exogenous PtdSer, we examined the effect of exogenous PtdSer on the PtdSer biosynthesis in a mutant of CHO-K1 cells, PSA-3, which lacks PSS I but has normal PSS II activity (1, 2). The PtdSer synthesis through the action of PSS II in the mutant cells was previously shown to increase upon cultivation with exogenous ethanolamine (8). Therefore, the culture medium supplemented with ethanolamine, in addition to the normal culture medium without ethanolamine, was used to examine the effect of exogenous PtdSer on the PtdSer biosynthesis in PSA-3 mutant and CHO-K1 cells. As shown in Fig. 2, in both the medium supplemented with and without ethanolamine, the PtdSer formation in CHO-K1 cells was reduced by ~95% upon addition of PtdSer to the medium, as measured as the incorporation of L-[14C]serine into PtdSer. Similarly, the PtdSer formation in the PSS I-defective PSA-3 mutant was reduced by ~95% upon addition of PtdSer to the medium supplemented with and without ethanolamine (Fig. 2). These results confirmed that the PtdSer formation by PSS II is inhibited upon addition of PtdSer to the culture medium.

PtdSer Biosynthesis in CHO-K1 Cells Transfected with the Wild-type and R97K Mutant pssB cDNA—PSS II has an arginine residue at position 97 (8), which corresponds to Arg-95 of PSS I identified as a critical residue for the control of PSS I activity (11). To determine whether Arg-97 of PSS II is involved in the control of PSS II activity, we constructed a R97K mutant pssB cDNA and transfected CHO-K1 cells with each of plasmids pSVpssB/neo and pSVR97K-pssB/neo, which carry, respectively, the wild-type and R97K mutant pssB cDNAs in addition to a G418-resistant gene. From the resultant G418-resistant transformants, a pSVpssB/neo-transformed clone (designated as K1/wt-pssB) and a pSVR97K-pssB/neo-transformed clone (designated as K1/R97K-pssB) were purified and subjected to biochemical characterization. Cell homogenates of the K1/wt-pssB and K1/R97K-pssB transformants exhibited, respectively, 3.5- and 3.4-fold higher serine base exchange activity than that in the homogate of CHO-K1 cells. Because the serine base exchange in the homogate of CHO-K1 cells is catalyzed by PSS I and PSS II, each of which accounts for approximately 50% of the total serine base exchange activity in the homogate (2), this result suggested that the PSS II activity in the homogate of both the transformants was ~6-fold that in CHO-K1 cells. On cultivation in the medium without exogenous PtdSer, the PtdSer biosynthetic activity in K1/wt-pssB transformant was similar to that in CHO-K1 cells (Fig. 3). In contrast to the K1/wt-pssB transformant, the K1/R97K-pssB transformant exhibited ~4-fold higher PtdSer biosynthetic activity than that in CHO-K1 cells (Fig. 3), indicating that Arg-97 of PSS II is involved in the control of PSS II activity. Although the PtdSer biosynthesis in CHO-K1 cells was almost completely inhibited upon addition of PtdSer to the culture medium at the concentration of 100 μM, the PtdSer biosynthesis in the K1/wt-pssB transformant was reduced by only 35% upon addition of PtdSer (Fig. 3). Thus, elevation of the wild-type PSS II level appeared to affect the exogenous PtdSer-mediated inhibition of PtdSer biosynthesis. Unlike the PtdSer biosynthesis in CHO-K1 cells and the K1/wt-pssB transformant, the PtdSer biosynthesis in the K1/R97K-pssB transformant was not inhibited at all but elevated by the addition of PtdSer (Fig. 3), implying that Arg-97 of PSS II is involved in the exogenous PtdSer-mediated inhibition of PtdSer biosynthesis.

Phospholipid Compositions of the K1/wt-pssB and K1/R97K-pssB Transfomers—To examine the effects of elevation of the wild-type PSS II level and production of R97K mutant PSS II on the steady-state level of cellular PtdSer, we determined the phospholipid compositions of CHO-K1, K1/wt-pssB, and K1/R97K-pssB cells cultivated in the medium supplemented with or without 50 μM PtdSer. In the medium without exogenous PtdSer, the phospholipid composition of the K1/wt-pssB transformant was similar to that of CHO-K1 cells (Table I). In the medium supplemented with PtdSer, however, this transformant exhibited a 1.6-fold higher PtdSer level than that in CHO-K1 cells (Table I). This result suggested that elevation of the wild-type PSS II level affects homeostasis of the cellular PtdSer level in the medium supplemented with exogenous PtdSer. In contrast to the K1/wt-pssB transformant, even in the medium without exogenous PtdSer the K1/R97K-pssB transformant exhibited a 1.6-fold higher PtdSer level than that in CHO-K1 cells (Table I), implying that Arg-97 of PSS II is involved in homeostasis of the cellular PtdSer level. When
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Cells were seeded at ~1.5 × 10^6 cells/150-mm-diameter dish in the growth medium without or with 50 μM PtdSer at 37 °C. After 3 days, the cellular phospholipids were extracted and separated by two-dimensional thin layer chromatography as described (9). To quantitate the individual phospholipids, the phosphatate in each spot on a chromatogram was determined chemically (16). Two independent experiments gave similar results, one set of results being presented here. PS, phosphatidylethanolamine; PE, phosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol.

| Strain       | PtdSer supplementation | PS | PE | PC | SM | PI | Other |
|--------------|------------------------|----|----|----|----|----|-------|
| CHO-K1       | -                      | 7.1| 16.1| 58.4| 8.9| 6.7| 2.8  |
| K1/wt-pssB   | -                      | 7.7| 16.3| 56.1| 9.8| 6.0| 4.1  |
| K1/R97K-pssB | -                      | 11.4| 20.4| 52.4| 8.9| 2.8| 4.2  |
| CHO-K1       | +                      | 8.9| 21.4| 51.4| 10.0| 4.8| 3.4  |
| K1/wt-pssB   | +                      | 14.6| 18.0| 53.0| 8.0| 2.3| 4.1  |
| K1/R97K-pssB | +                      | 18.5| 16.0| 56.8| 3.8| 1.7| 3.2  |

**TABLE I** Phospholipid compositions of CHO-K1, K1/wt-pssB, and K1/R97K-pssB cells

**FIG. 4.** Effect of PtdSer on the serine base exchange activity in vitro. CHO-K1, K1/wt-pssB, and K1/R97K-pssB cells were seeded at ~1.5 × 10^6 cells/150-mm-diameter dish in the growth medium without exogenous PtdSer at 37 °C. PSA-3 cells were seeded under the same conditions except that the growth medium was supplemented with 30 μM PtdSer. After 3 days, the cells were washed twice with phosphate-buffered saline, and then a membrane fraction of each strain was prepared as described (11) and assayed for serine base exchange activity as described (1) in the presence of various amounts of PtdSer liposomes. The results are expressed as the percentage of activity relative to the specific activity of each strain, measured without exogenous PtdSer. The specific activities in the membrane fractions from CHO-K1, PSA-3, K1/wt-pssB, and K1/R97K-pssB cells, measured without exogenous PtdSer, were 8.4, 6.2, 20.5, and 15.9 mmol/h/mg protein, respectively. Values are the averages for duplicate assays with variation of <15% between duplicates. □, CHO-K1; ○, PSA-3; ◆, K1/ctl-pteB; ●, K1/R97K-pssB.

Next, we examined the PtdSer biosynthesis in PSB-2/R97K-pssB and PSB-2/pssB transformants and PSA-3 and PSB-2 mutants cells cultivated in the ethanolamine-containing medium supplemented with or without exogenous PtdSer. In the medium without exogenous PtdSer, the PtdSer biosynthetic activities in the PSB-2/R97K-pssB and PSB-2/pssB transformants were, respectively, ~4- and ~2-fold that in the PSB-2 mutant (Fig. 5B), indicating that PSS II encoded by the C-terminal transformant (designated as PSB-2/R97K-pssB), the PSB-2/pssB transformant, which had been obtained by the transfection of PSB-2 mutant cells with the wild-type pssB cDNA and shown to have a normal level of PSS II activity (2), was biochemically characterized with respect to the PtdSer synthesis in a cell-free system and in intact cells. The serine base exchange activities in membrane fractions of the PSB-2/ R97K-pssB and PSB-2/pssB transformants were, respectively, 7.8- and 5.5-fold that in the PSB-2 mutant (Fig. 5A).
transfected cDNAs contributes to PtdSer biosynthesis in the transformants. Compared with the PSA-3 mutant, the PSB-2/R97K-pssB transformant but not the PSB-2/pssB transformant exhibited a significant (2.4-fold) increase in the PtdSer biosynthetic activity, in the medium without exogenous PtdSer (Fig. 5B). Upon cultivation with exogenous PtdSer, the PtdSer biosynthesis in the PSB-2/pssB transformant was inhibited by >95%, like that in the PSA-3 and PSB-2 mutants (Fig. 5B). In contrast, the PtdSer biosynthesis in the PSB-2/R97K-pssB transformant was highly resistant to this inhibition by exogenous PtdSer and was reduced by only ~15% upon addition of PtdSer (Fig. 5B). These results obtained in the experiments involving the PSB-2/R97K-pssB and PSB-2/pssB transformants showed that Arg-97 of PSS II is critical for the exogenous PtdSer-mediated inhibition of PSS II.

**DISCUSSION**

PtdSer in CHO cells is synthesized through the action of PSS I and II, which catalyze the exchange of α-serine with the base moiety of PtdCho and PtdEtn, respectively (2–4). The cDNAs of both the PSS I and PSS II genes named pssA and pssB, respectively, have been isolated (6–8), and the cDNA of PtdSer has been successfully utilized for studying the control of PSS I activity in CHO-K1 cells (11). The present study focused upon the control of PSS II activity. The conversion of exogenous PtdEt to PtdSer, which is catalyzed by PSS II, in CHO-K1 cells is reduced by >90% upon addition of PtdSer to the culture medium. The PtdSer formation in the PSS I-defective PSA-3 mutant, as well as that in CHO-K1 cells, is depressed by >95% upon addition of PtdSer to the culture medium. Furthermore, PtdSer inhibits almost completely the serine base exchange activity for PtdSer synthesis in membrane fractions of PSA-3 mutant and CHO-K1 cells. These results indicate that the PtdSer biosynthesis through the action of PSS II is inhibited by exogenous PtdSer.

The PtdSer biosynthesis in a PSS II-overproducing stable transformant of CHO-K1 cells, K1/wt-pssB, is reduced by only ~35% upon addition of PtdSer to the culture medium, whereas the PtdSer biosynthesis in CHO-K1 cells is reduced by >95% upon addition of PtdSer. When cultivated in the medium supplemented with exogenous PtdSer, K1/wt-pssB cells exhibit significant (1.6-fold) elevation of the cellular PtdSer level. Furthermore, the serine base exchange activity in the membrane fraction of K1/wt-pssB cells is not inhibited by PtdSer at all, in contrast to the complete inhibition of the exchange activity in the CHO-K1 membrane. Therefore, the elevation of the PSS II level in CHO-K1 cells leads to the loss of normal control of PSS II activity by exogenous PtdSer. Why does the elevation of the PSS II level affect the exogenous PtdSer-mediated inhibition of PSS II? One feasible explanation is that the inhibition of PSS II by exogenous PtdSer requires an unknown factor that mediates this inhibition, and then the elevation of the PSS II level results in a deficiency of this factor. Confirmation of this speculation awaits the purification or cloning of such a putative inhibition-mediating factor.

Although the serine base exchange activity in the membrane of PSS II-overproducing K1/wt-pssB cells is not inhibited by exogenous PtdSer, the exchange activity in a membrane fraction of a PSS I-overproducing stable transformant of CHO-K1 cells, K1/wt-pssA, is inhibited by exogenous PtdSer (11). The PtdSer biosynthesis in K1/wt-pssA cells is inhibited by >90% upon addition of PtdSer to the culture medium (11), whereas the biosynthesis in K1/wt-pssB cells is highly resistant to this inhibition by exogenous PtdSer. Therefore, in contrast to overproduced PSS II, overproduced PSS I appears to be inhibited by exogenous PtdSer. One possible explanation for these observations is that the inhibition of PSS I occurs through the direct binding of exogenous PtdSer to PSS I, whereas the inhibition of PSS II by exogenous PtdSer requires the putative inhibition-mediating factor saturable with overproduced PSS II. Given that the inhibition of both PSS I and PSS II by exogenous PtdSer requires the putative inhibition-mediating factor, the inhibition of PSS II by exogenous PtdSer might be mediated by a relatively abundant factor that is different from the putative inhibition-mediating factor for PSS II.

The elevation of the wild-type PSS II level leads to the loss of normal control of PSS II by exogenous PtdSer, as described above; nevertheless, in the medium without exogenous PtdSer, K1/wt-pssB cells with elevated PSS II activity exhibit a normal PtdSer biosynthetic rate and cellular PtdSer level, similar to those in CHO-K1 cells. The maintenance of a normal PtdSer biosynthetic rate in K1/wt-pssB cells is probably not due to limitation of precursor molecules of PtdSer, because, in contrast to K1/wt-pssB cells, another stable transformant, K1/R97K-pssB, which appears to express R97K mutant PSS II at a level similar to that of wild-type PSS II in K1/wt-pssB cells, exhibits a ~4-fold higher PtdSer biosynthetic rate and a 1.6-fold higher cellular PtdSer level, relative to those in CHO-K1 cells, in the medium without exogenous PtdSer. These results suggest that for maintenance of the normal PtdSer biosynthetic rate, the activity of overproduced PSS II in K1/wt-pssB cells is depressed through post-translational mechanisms other than those for the exogenous PtdSer-mediated inhibition. Furthermore, the results indicated that Arg-97 of PSS II is a critical residue for this depression of overproduced PSS II activity.

Arg-97 of PSS II appears to be a critical residue also for the exogenous PtdSer-mediated inhibition of PSS II, as judged from the following results: 1) the PtdSer biosynthesis in K1/R97K-pssB cells was not inhibited by the addition of PtdSer to the medium at all, whereas the PtdSer biosynthesis in K1/wt-pssB cells was not completely but was significantly inhibited upon addition of PtdSer; 2) when the cDNA-directed R97K mutant and wild-type PSS II activities were expressed at non-overproduction levels in a PSS I- and PSS II-defective PSB-2
mutant, the serine base exchange activity in a membrane fraction of the mutant PSS II-expressing PSB-2 mutant, PSB2/R97K-pssB, was not significantly affected by the addition of PtdSer to the assay mixture, whereas that of the wild-type PSS II-expressing PSB-2 mutant, PSB-2/pssB, was inhibited by ~90% upon addition of PtdSer; and 3) although the PtdSer formation in PSB-2/pssB cells is almost completely inhibited upon addition of PtdSer to the culture medium, the PtdSer formation in PSB-2/R97K-pssB cells is reduced by only ~15% upon addition of PtdSer.

In summary, the results presented in this report indicated that PSS II in CHO-K1 cells is inhibited by exogenous PtdSer and that the activity of overproduced PSS II in CHO-K1 cells is depressed for maintenance of the normal PtdSer biosynthetic rate, probably through molecular mechanisms different from those for the exogenous PtdSer-mediated inhibition. Furthermore, the Arg-97 of PSS II was shown to be a critical residue for both the exogenous PtdSer-mediated inhibition of PSS II and the depression of overproduced PSS II activity.

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