Roles of Rho-associated Kinase and Myosin Light Chain Kinase in Morphological and Migratory Defects of Focal Adhesion Kinase-null Cells*

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Fibroblasts derived from focal adhesion kinase (FAK)-null mouse embryos have a reduced migration rate and an increase in the number and size of peripherally localized adhesions (Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., and Yamamoto, T. (1995) Nature 377, 539–544). In this study, we have found that Y27632, a specific inhibitor for Rho-associated kinase (Rho-kinase), dramatically reversed the round cell morphology of FAK−/− cells to a spread fibroblast-like shape in 30 min and significantly enhanced their motility. The effects of Y27632 on the FAK−/− cell morphology and motility were concomitant with reorganization of the actin cytoskeleton and redistribution of focal adhesions. Conversely, the expression of the constitutively active Rho-kinase in FAK+/+ cells led to round cell shape and inhibition of cell motility. Furthermore, coincident with the formation of cortical actin filaments, myosin light chain (MLC), Ser-19-phosphorylated MLC, and MLC kinase mainly accumulated at the FAK−/− cell periphery. We found that the disruption of actin filaments by cytochalasin D prevented the peripheral accumulation of MLC kinase and that inhibition of myosin-mediated contractility by 2,3-butanedione monoxime induced FAK−/− cells to spread. Taken together, our results suggest that Rho-kinase may mediate the formation of cortical actomyosin filaments at the FAK−/− cell periphery, which further recruits MLC kinase to the cell periphery and generates a non-polar contractile force surrounding the cell, leading to cell rounding and decreased motility.

Rho GTPases including Rho, Rac, and Cdc42 are key modulators of the actin cytoskeleton (1–3). They are critical for the cell shape changes and adhesion dynamics that drive cell migration (4–7). Among the Rho GTPase family, Rho induces the formation of focal adhesions and stress fibers (7, 8). Interestingly, although a basal level of Rho activity is needed for fibroblast migration, too much Rho activity impede migration (4, 9, 10). It has been shown that the concerted action of two of the immediate Rho targets, Rho-associated kinase (Rho-kinase)3/ROCK and the formin homology protein mDia1, mediate the effect of Rho on matrix adhesion and the actin cytoskeleton (11). In particular, Rho-kinase was shown to stimulate myosin-driven contractility in smooth muscle and nonmuscle cells by phosphorylating, thereby inactivating myosin light chain (MLC) phosphatase (12, 13), and possibly by direct phosphorylation of MLC (14–16). In addition to Rho-kinase, MLC kinase (MLCK) is another kinase that phosphorylates the MLC in both smooth muscle and nonmuscle cells (17, 18). The phosphorylation of MLC on Ser-19 and to a lesser extent on Thr-18 by MLCK promotes the assembly of myosin filaments and activates its ATPase activity, which stabilizes the actin-myosin interaction and promotes cell contractility (19–22). Recently, Rho-kinase and MLCK were suggested to play distinct roles in spatial regulation of MLC phosphorylation. Rho-kinase appears to be important for MLC phosphorylation in the center of cells, and MLCK is responsible for phosphorylating MLC at the cell periphery (16).

Focal adhesion kinase (FAK), a 125-kDa cytoplasmic tyrosine kinase localized in focal contacts, has been known to play an important role in integrin-mediated cell migration (23). Fibroblasts derived from FAK-null mouse embryos are more rounded and poorly spread than their wild-type counterparts (23). They show an overabundance of focal adhesions, enriched cortical actin filaments at the cell periphery, and a decreased migration rate (23, 24). It has been suggested that the increase in peripheral adhesions results from an inhibition of turnover in FAK−/− cells (23), which may result from constitutive activation of Rho (24). Because of the known involvement of Rho-kinase and MLCK in cell contractility, a major factor controlling cell migration, we hypothesize that abnormal regulation of Rho-kinase and MLCK may underlie the migratory defect of FAK−/− cells.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum, non-essential amino acids, sodium pyruvate, and 2-mercaptoethanol were purchased from Invitrogen. Y27632, a specific inhibitor of Rho-kinase, was purchased from Calbiochem. The monoclonal anti-MLCK, monoclonal anti-MLC, monoclonal anti-α-tubulin, bovine MLC, myelin basic protein (MBP), cytochalasin D, and 2,3-butanedione monoxime (BDM) were purchased from Sigma-Aldrich. The polyclonal anti-Rho-kinase and monoclonal anti-paxillin were purchased from Transduction Laboratories (Lexington, KY). The plasmid pEGFP-N1-MLCK and polyclonal anti-MLC were described previously (25). The plasmid pEGFP-N1-MLCK and polyclonal anti-MLC were described previously (25). The plasmid pEGFP-N1-MLCK and polyclonal anti-MLC were described previously (25). The plasmid pEGFP-N1-MLCK and polyclonal anti-MLC were described previously (25).

Cell Culture and Transfections—FAK+/+ and FAK−/− cells were light chain; MLC, MLC kinase; MBP, myelin basic protein; BDM, 2,3-butanedione monoxime; DIC, differential interference contrast; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein.

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Wound Healing Assays and Time-lapse Microscopy—FAK−/− cells were grown on glass coverslips with 0.17 mm in thickness and 42 mm in diameter. The monolayer of cells was wounded by manual scratching with a pipette tip and then treated with or without 10 μM Y27632. Cells on the microscope stage were maintained at 37 °C with a humid CO2 atmosphere in a microcultivation system (model POC-R, Zeiss) with temperature and CO2 control devices (Tempcontrol 37-2 digital and CTI controller 3700 digital, Zeiss). Cells were monitored under differential interference contrast (DIC) optics on an inverted Zeiss microscope (Axiovert 100) using Zeiss 40X LD-Apochromat objective. Time-lapse sequential micrographs were captured per minute using a cooled CCD camera (CoolSNAP fx, Roper Scientific, Inc) and analyzed by Meta Imaging Series™ software (version 4.5) from Universal Imaging Corporation (West Chester, PA).

Fluorescence Microscopy—Cells were plated on 13-mm glass coverslips for 24 h, washed three times with phosphate-buffered saline, fixed for 10 min with phosphate-buffered paraformaldehyde, and permeabilized in phosphate-buffered saline containing 0.2% Triton X-100 for 10 min. Coverslips were stained with primary antibodies for 60 min and followed by goat anti-mouse TRITC or fluorescein isothiocyanate (FITC) conjugated secondary antibodies (Jackson Immunoresearch laboratories) at 4 μg/ml for 60 min. All antibodies used in immunofluorescence staining in this report are monoclonal including anti-paxillin (1:100), anti-MLC (1:50), anti-MLC (1:100), anti-Ser-19-phosphorylated MLC (1:100), and anti-β-tubulin (1:100). TRITC-conjugated phalloidin (Sigma-Aldrich) at 2 μM was used to stain actin filaments. Coverslips were mounted in anti-fading solution and viewed using a Zeiss LSM-510 laser-scanning confocal microscope image system with a Zeiss 100X Plan-Apochromat objective (NA 1.4 oil).

Immunoprecipitations, Immunoblotting, and in Vitro Kinase Assays—Cells were lysed with 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10 mM glycerol, and 1 mM Na3VO4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.2 trypsin inhibitory units/ml aprotinin, and 20 μg/ml leupeptin). The lysates were centrifuged for 10 min at 4 °C to remove debris, and the protein concentrations were determined using the Bio-Rad Protein assay. The aliquots of lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Schleicher & Schuell). Immunoblotting was performed with polyclonal anti-Rho-kinase or an antibody specific for -tubulin (Sigma-Aldrich) at 1 μg/ml, polyclonal anti-MLC (1:1000), polyclonal anti-Myc (1:1000) using the Amersham Biosciences enhanced chemiluminescence system for detection.

For MLCK activity assays, cells lysates were incubated with 2.5 μl of monoclonal anti-MLCK for 1.5 h at 4 °C. Immunocomplexes were collected on protein A-Sepharose beads coupled with rabbit anti-mouse IgG. The beads were washed three times with 1% Nonidet P-40 lysis buffer and one time with 20 mM Tris, pH 7.4. Kinase reactions were carried out in 40 μl of kinase buffer (20 mM Tris, pH 7.4, 10 mM MgCl2, 10 mM CaCl2, 2 mM dithiothreitol, and 0.1 μM calmodulin) containing 10 μCi of [γ-32P]ATP and 10 μg of bovine MLC or MBP for 20 min at 25 °C. For Rho-kinase activity assays, cells lysates were incubated with 5 μl of polyclonal anti-Rho-kinase for 1 h at 4 °C. The immunocomplexes were washed and subjected to kinase reaction in 40 μl of kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl2, 3 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA) in the presence of 10 μCi of [γ-32P]ATP and 10 μg of MBP. Reactions were terminated by the addition of SDS sample buffer, and proteins were resolved by SDS-polyacrylamide gel electrophoresis.

Statistics—Statistical analyses were performed with a Student’s t test. Differences were considered to be statistically significant at p < 0.05.

RESULTS

The constitutive activation of Rho has been found to correlate with the inhibition of focal adhesion turnover in FAK−/− cells (24). Accordingly, we found that the activity of Rho-kinase, an immediate downstream target of Rho, in FAK−/− cells was ~30% higher than that in FAK+/+ cells (Fig. 1). To examine whether Rho-kinase mediates the effect of constitutively active Rho on the morphology of FAK−/− cells, a specific inhibitor for Rho-kinase, Y27632, was employed (28). This pharmacological reagent was reported to be specific for Rho-kinase at 10 μM (29).

For other reports in the literature (16, 30), Y27632 at this concentration reduced the formation of actin stress fibers in FAK−/− cells, which became more flattened and unable to move (data not shown), supporting an essential role of Rho-kinase in stress fiber formation and cell motility. For FAK−/− cells, Y27632 promptly altered their rounded morphology to a spread fibroblast-like shape in 30 min (Fig. 2A) and significantly enhanced their motility (Fig. 3). Four hours after the removal of Y27632 from the medium, the cells became less spread and finally rounded (Fig. 2B) accompanied with the recovery of Rho-kinase activity (data not shown). Importantly, the spreading of FAK−/− cells induced by Y27632 was concomitant with marked reorganization of the actin cytoskeleton from cortical actin bundles into long parallel filaments similar to those seen in polar migratory fibroblasts (Fig. 2C). In addition, paxillin, a protein localized in focal adhesions, was found from peripheral patchlike to scattered dotlike distribution upon the addition of Y27632 (Fig. 2C). These results suggest that in FAK−/− cells, the constitutive activation of Rho-kinase may be involved in the formation of cortical actin structures, abundance of peripheral adhesions, and rounded cell shape.

It is worth noting that the Y27632-treated FAK−/− cells exhibited not only spread cell shape but also characteristics of motile cells including membrane ruffles and the formation of filopodia and lamellipodia (Figs. 2 and 3). To examine the effect of Y27632 on the motility of FAK−/− cells, wound healing assays were performed and monitored by time-lapse microscopy. We found that the motility of FAK−/− cells was indeed enhanced by Y27632 at 10 μM. Using an image analysis software, the motility of FAK−/− cells was measured at an average speed of 5 μm h−1 in the absence of Y27632 and an average speed of 20 μm h−1 in the presence of Y27632 (Fig. 3). These results suggest that constitutive activation of Rho-kinase may...
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Fig. 2. The specific Rho-kinase inhibitor Y27632 induces FAK−/− cells to spread accompanied by reorganization of actin cytoskeleton and redistribution of focal adhesions. A, FAK−/− cells were sparsely grown on glass overnight and then treated with or without 10 μM Y27632. 30 min later, micrographs were taken by a cooled CCD under a differential interference contrast (DIC) microscope. Bar, 30 μm B, FAK−/− cells were treated with 10 μM Y27632 for 30 min to induce their spreading. The medium was then changed by fresh medium. After 4 h, micrographs were taken. The two micrographs in B represent the morphological change of the same cell. Bar, 10 μm. C, FAK−/− cells were treated with or without 10 μM Y27632 for 30 min and then stained for actin with fluorescein isothiocyanate (FITC)-phalloidin and for paxillin with monoclonal anti-paxillin. Bar, 10 μm.

Fig. 3. Y27632 enhances the motility of FAK−/− cells. FAK−/− cells were grown into monolayer on glass. The monolayer of cells was wounded by manual scratching with a pipette tip and then treated with or without 10 μM Y27632. The time-lapse micrographs were taken every 1 min for 4 h to record the healing process. The representative micrographs at 0, 2, and 4 h are shown. An average migratory speed of 10 cells at the front was measured by Meta Imaging Series software, version 4.5. Values (mean ± S.E.) are from three independent experiments. Bar, 30 μm.
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Fig. 4. Expression of constitutively active Rho-kinase leads to round cell shape of FAK+/+ cells. A, FAK+/+ cells were transiently transfected with a plasmid encoding GFP or co-transfected with two plasmids encoding GFP and the Myc epitope-tagged constitutively active Rho-kinase (CA Rho-kinase), respectively. GFP serves as an indicator for transfected cells. 6 h after transfection, cells were visualized under a DIC/fluorescent microscope. B, cell lysates from the cells as described in A were analyzed by immunoblotting with monoclonal anti-Myc to verify the expression of CA Rho-kinase. Bar, 10 μm.

(indicated by paxillin localization), they had no stress fiber-like actin filaments in the center of the cells (Fig. 6B).

Rho-kinase and MLCK have been suggested to play distinct roles in spatial regulation of MLC phosphorylation (16). To investigate which kinase is responsible for phosphorylating MLC at the FAK−/− cell periphery, the subcellular localizations of Rho-kinase and MLCK were examined. Unfortunately, because of the failure of the anti-Rho-kinase antibody in immunofluorescence staining, we were unable to determine the localization of Rho-kinase in these cells. However, the localization of MLCK in FAK−/− cells was unusual, which was strongly accumulated at the cell periphery instead of a diffuse distribution as seen in FAK+/+ cells (Fig. 7A). To further confirm this phenomenon, GFP-MLCK was transiently expressed in FAK+/+ and FAK−/− cells, and the fluorescence of GFP-MLCK was visualized in living cells (Fig. 7A). Similar to endogenous MLCK in FAK−/− cells, GFP-MLCK was mainly accumulated at the cell periphery. In FAK−/− cells, GFP-MLCK distributed both at cell periphery and the center of the cell where it assembled into long filaments. The expression and activity of MLCK was next compared between FAK−/− and FAK+/+ cells (Fig. 7B). Surprisingly, the amount of MLCK in FAK−/− cells was ~3-fold of that in FAK+/+ cells, which was approximately correlated with the difference in MLCK activity between these two cell lines. Thus, although we cannot exclude the role of Rho-kinase in MLC phosphorylation at the FAK−/− cell periphery, the peripheral localization and high expression of MLCK in these cells render it more likely to be involved in this event.

MLCK has been reported to directly interact with actin filaments (34). To examine whether the formation of the cortical actin cytoskeleton is required for peripheral accumulation of MLCK, FAK−/− cells were treated with 2.5 μM cytochalasin D for 1 h to disrupt actin cytoskeleton and then co-stained for actin and MLCK (Fig. 8A). In the control experiments, GFP-MLCK was expressed in FAK−/− cells, and its peripheral localization was visualized after treatment of 30 μM nocodazole, which disrupts microtubules (Fig. 8B). Our results clearly demonstrated that the disruption of cortical actin cytoskeleton but not microtubule prevented the peripheral accumulation of MLCK in FAK−/− cells, suggesting that the formation of cortical actin bundles is essential for MLCK accumulation at the cell periphery.

DISCUSSION

In addition to genetic approaches with dominant negative or positive mutants of Rho-kinase, a pharmacological approach using the Rho-kinase inhibitor Y27632 has frequently been used to examine the role of Rho-kinase in cellular functions. So far, Rho-kinase has been shown to be involved in the formation of stress fibers and focal adhesions (35, 36) and in various contractile processes including cell motility (37), smooth muscle contraction (12, 14, 15), neurite retraction (38), tail retraction of migrating monocytic (39), and cytokinesis (40). In the literature we have searched, it appears that Y27632 consistently has a negative impact on the above cellular functions. Unexpectedly, we found in this study that Y27632 has a positive impact on FAK−/− cells that promptly induces them to spread and facilitates their motility (Figs. 2A and 3). Together
with the results that the activity of Rho-kinase in FAK−/− cells is higher than that in FAK+/+ cells (Fig. 1) and that the expression of the constitutively active Rho-kinase in FAK+/+ cells causes cell rounding and inhibition of cell motility (Fig. 4), it is possible that the morphological and migratory defects of FAK−/− cells may result from the constitutive activation of Rho-kinase. These data also implicate that too much Rho-kinase activity may cause cell rounding and impaired cell motility. Accordingly, it has recently been found that high Rho activity is required to maintain the round cell shape of undifferentiated embryonic mesenchymal cells (41).

Our finding that Y27632 enhanced stress fiber formation in FAK−/− cells (Fig. 2C) is somewhat a discrepancy to other reports in which Y27632 was shown to inhibit stress fiber formation (16, 30). Although the reason for this discrepancy is unclear, the LIM kinase/cofilin pathway might be involved. LIM kinase is known to be phosphorylated by Rho-kinase, which in turn is activated to phosphorylate cofilin (16, 42). Cofilin is an actin-depolymerizing factor that binds to monomeric actin as well as filamentous actin (43). The phosphorylation of cofilin at Ser-3 by LIM kinase reduces its actin binding and depolymerizing activities, which is thought to contribute to Rho-induced stress fiber formation (42, 44). It appears that only a limited amount of cofilin is phosphorylated in response to extracellular stimuli such as lysophosphatidic acid, which induce changes in cytoskeletal organization (30). However, Zebda et al. (45) showed that the phosphorylation of the majority of the cell cofilin by expressing the kinase domain of LIM kinase completely inhibits the generation of actin-barbed ends at the tip of the leading edge and lamellipodial extension in response to epidermal growth factor. Therefore, it is possible that constitutive activation of Rho-kinase in FAK−/− cells may lead to increased levels of phosphorylated cofilin analogous to that reported by Zebda et al. (45). The suppression of Rho-kinase activity by Y27632 in FAK−/− cells may reduce the amount of phosphorylated cofilin to an optimal level, thus allowing for the formation of actin-barbed ends required for lamellipodial protrusion.

Another major finding in this report is that active actomyosin filaments are actually assembled at the FAK−/− cell periphery characterized by peripheral accumulations of MLC, Ser-19-phosphorylated MLC, and MLCK (Figs. 5 and 7). We further demonstrated that the accumulation of MLCK at the FAK−/− cell periphery depends on the formation of cortical actin structures (Fig. 8). In addition, the myosin ATPase inhibitor BDM, which abolishes actomyosin-driven contractility, was found to induce FAK−/− cell spreading (Fig. 6), supporting the idea that the round morphology of FAK−/− cells may be caused by a global contraction at the cell periphery. Therefore, our results in this study together suggest that the constitutive activation...
of Rho-kinase may first induce the formation of cortical actin bundles, which subsequently recruits the binding of myosin II and MLCK, thereby leading to MLC phosphorylation and cell contraction. Because the actomyosin filaments are distributed around the FAK−/− cell, the contractile forces driven by these structures are probably centripetal, which may result in cell rounding and a deficiency in cell migration. This model provides a plausible explanation for the morphological and migratory defects of FAK−/− cells. It is worth noting that in addition to its peripheral accumulation, the elevated level of MLCK may also contribute to strong centripetal contraction of FAK−/− cells. The possibility that FAK modulates the expression and/or activity of MLCK remains to be tested.

In this study, we found that the high Rho-kinase activity appears to be correlated with an increase in the number and size of peripherally localized adhesions and enriched cortical actin bundles in FAK−/− cells. Although the underlying mechanism for such phenomena is currently unknown, the phosphorylation of two cytoskeletal proteins, adducin and moesin, by Rho-kinase may be involved (37, 46–48). Adducin is known to facilitate the interaction between F-actin and spectrin to form a cortical membrane skeletal meshwork (49). Moesin belongs to a family of three closely related proteins named ERM for ezrin/radixin/moesin. These proteins are found in actin-rich cell surface structures where they function as bridges between the plasma membrane and the actin filaments (50). The phosphorylation of adducin and moesin by Rho-kinase was reported to enhance their interactions with F-actin (46, 48, 51), which may subsequently facilitate the formation of cortical actin filaments as seen in FAK−/− cells. Alternatively, the formation of the cortical actin structure may be attributed to the abnormal regulation on the phosphorylation of α-actinin in FAK−/− cells. Because of its localization in focal adhesions, α-actinin was suggested to anchor the actin filaments to the plasma membrane (52). Recently, FAK was found to phosphorylate α-actinin, thereby reducing the interactions between α-actinin and actin filaments (53). A decrease in the affinity of α-actinin for actin resulting from phosphorylation by FAK could facilitate the turnover of focal adhesions as a result of diminished contact with the cytoskeleton. Thus, it is possible that FAK deficiency may maintain α-actinin at a low tyrosine phosphorylation status, thereby stabilizing the interaction between α-actinin and actin filaments at the plasma membrane. Experiments to examine these possibilities are in progress.

Although BDM treatment allowed FAK−/− cells to spread presumably by the relief of peripheral contraction, it failed to promote their motility (Fig. 6). In contrast to Y27632-treated FAK−/− cells, the BDM-treated cells completely lose their ability to move and divide. In fact, it is not surprising to observe such consequences, because actomyosin-based contractility is known to be essential for cell migration and division. However, it is more interesting to note that there were no or very few long parallel actin filaments assembled in the center of the BDM-treated cells, supporting the idea that cell contractility is required for the formation and/or maintenance of stress fibers. In addition, we found that paxillin-enriched focal adhesions were maintained in the BDM-treated cells, suggesting that myosin-based contractility may not be essential for the initial assembly and maintenance of focal adhesions.

The effect of Y27632 on the morphology and motility of FAK−/− cells is prompt and reversible. In particular, Y27632 induces a prominent reorganization of the actin cytoskeleton and redistribution of focal adhesions in these cells (Fig. 2). With these properties, the migration of FAK−/− cells induced by Y27632 as described in this report might become a useful model to study the mechanisms of cell migration in the absence of FAK expression. For example, the dynamics of the actin cytoskeleton and focal adhesions may be visualized in living migratory FAK−/− cells by expression of GFP-tagged molecules such as actin, paxillin, and α-actinin. Moreover, cell migration is already known to involve a series of complex signaling cascades and the coordinated regulation of many cytoskeletal proteins. How the suppression of Rho-kinase activity by Y27632 affects other molecules and finally turns on the “migration machinery” remains to be investigated. For example, Y27632-treated FAK−/− cells exhibit typical characteristics of motile cells such as active membrane ruffling and filopodia formation. Because Rho family GTPases Rac and Cdc42 are known to participate in these events (7, 8), it is probable that the suppression of Rho-kinase by Y27632 may result in the activation of both molecules in FAK−/− cells. In fact, we have found that Rac is actually activated upon Y27632 addition in FAK−/− cells.2 This observation is in agreement with the notion that blocking one Rho GTPase protein pathway might result in the modulation of other GTPases affecting cell migration and morphology (54, 55). In particular, Rho and Rac activities were

2 B.-H. Chen and H.-C. Chen, unpublished data.
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suggested to be mutually antagonistic (56, 57). Finally, the phenotypes of the fibroblasts deficient in the SHP-2 phosphatase are remarkably similar to those of FAK−/− cells, both of which have an increased number and size of adhesions and impaired spreading and migration (58). It will be of interest to examine whether Y27632 is capable of promoting spreading and motility of SHP-2−/− cells.

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