Attachment Organelle Formation Represented by Localization of Cytadherence Proteins and Formation of the Electron-Dense Core in Wild-Type and Mutant Strains of *Mycoplasma pneumoniae*

Shintaro Seto and Makoto Miyata*

Department of Biology, Graduate School of Science, Osaka City University,
Sumiyoshi-ku, Osaka 558-8585, Japan

Received 25 June 2002/Accepted 22 October 2002

Cytadherence proteins of *Mycoplasma pneumoniae* are localized at the attachment organelle, which is involved in adhesion, gliding motility, and cell division. The localization of these proteins in cytodherence-deficient mutants was examined by immunofluorescence microscopy. In the class I-2 mutant, which has a frameshift mutation in the *hmw2* gene, fluorescent foci for HMW1 and HMW3 were found with reduced intensity, and P1 adhesin showed reduced focusing. However, foci for P90, P40, P30, and P65 were not observed in this mutant. In the class IV-22 mutant, which lacks expression of P1, P90, and P40, the other cytodherence proteins (HMW1, HMW3, P30, and P65) were focused. In a mutant lacking HMW1, signals for HMW3, P90, P40, P30, and P65 were not found, and P1 was distributed throughout the cell. These results suggest that HMW1 is essential for the localization of all other cytodherence proteins, while HMW2 is essential for the localization of P90, P40, P30, and P65. The electron-dense core in cytodherence mutants was observed by thin-section electron microscopy, suggesting that its formation depends on HMW1 and HMW2 and that P1 localization occurs independent of the formation of the electron-dense core. Doubly stained preparations visualized by immunofluorescence microscopy showed that the P1 adhesin, P90, and P40 colocalized to a subregion of the attachment organelle in the wild-type strain. HMW1 and HMW3 also colocalized to a different subregion of the attachment organelle, while P30 and P65 localized at more distal ends of cell poles than HMW1 and HMW3. These differences were more pronounced in cytodherence mutants. These results suggest that there are three distinct subcellular protein localization sites in the attachment organelle, which were represented by HMW1-HMW3, P1-P90-P40, and P30-P65.

Mycoplasmas are commensal or parasitic bacteria that have small genomes and no peptidoglycan layer (31). Several mycoplasmas have terminal structures that enable them to adhere to the host cell surface for colonization and nutrient acquisition. The terminal structure is also thought to be involved in cell division and gliding motility (2, 6, 7, 14, 24–26a). The terminal structure of *Mycoplasma pneumoniae*, designated the attachment organelle, is featured with clustering of P1 adhesin (4, 8, 12, 30). Several proteins, including HMW1, HMW2, P90, P40, and P30, have also been shown to be essential for cytodherence by mutant studies, and HMW1, HMW2, HMW3, P90, P40, P30, and P65 have been reported to localize at the attachment organelle (2, 15, 16, 34). Examination of cytodherence protein localization in mutants can suggest the roles of proteins in the formation of the attachment organelle, but such studies have been limited to a few proteins because the studies have generally been done by using immunoelectron microscopy (11, 33, 37, 38). Immunofluorescence microscopy was recently applied to mycoplasmas and enabled quick localization of proteins (13, 34). In a previous study, we and other workers examined three mutants and showed that HMW1 is essential for localization of the other six cytodherence proteins (i.e., HMW3, P1, P30, P90,

P40, and P65), while mutations in P30, P90, and P40 did not affect localization much (34). If we performed similar analyses with a strain having a mutation in the gene encoding HMW2, a master protein controlling the stability of HMW1 and HMW3 (27), we could obtain valuable information about its roles in localization of other cytodherence proteins.

Thin-section electron microscopy has demonstrated that there is an “electron-dense core” in the attachment organelle of *M. pneumoniae* (5, 35, 39), which is believed to be identical to the rod structure that can be observed when mycoplasma cells are treated with detergent (10, 23, 32). This rod structure is thought to be composed of cytodherence-related proteins and additional proteins (32). However, little is known about the proteins responsible for its formation.

In this study, we determined the localization of cytodherence proteins by using immunofluorescence microscopy for five mutants, including mutants missing HMW2 or P1 adhesin, whose role in localization of other proteins is little known, and we also demonstrated that the electron-dense core is formed in various mutants by using thin-section electron microscopy.

MATERIALS AND METHODS

Strains and cultivation. Wild-type *M. pneumoniae* M129 and cytodherence mutants were grown in 25 ml of Aluotto medium (1) for 2 or 3 days at 37°C by using plastic culture flasks to a density of about 10⁷ to 10⁸ CFU/ml. Strains used in this study are summarized in Table 1. Frozen stocks of cytodherence mutants were checked for hemadsorption activity and were determined not to contain

* Corresponding author. Mailing address: Department of Biology, Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan. Phone: 81(6)6605 3157. Fax: 81(6)6605 3158. E-mail: miyata@sci.osaka-cu.ac.jp.

TABLE 1. Mutant strains used in this study

Strain	Missing or truncated proteins	Proteins with reduced amt	Mutated gene(s) ^a	Complementation test	Reference(s)
Class I-2	HMW2	HMW1, HMW3, P65	<i>hmw2</i> (F)	+ ^b	3, 9, 13, 17, 27
Class II-3	P30	P65	<i>p30</i> (F)	+	13, 17, 33
Class III-4	P90, P40	None	NR ^c	NR	13, 17
Class IV-22	P1, P90, P40	None	NR	NR	13, 17
M5	P90, P40	NR	NR	NR	18
M6	HMW1, P30	P65	<i>hmw1</i> (F), <i>p30</i> (D)	+	13, 20, 33
M7	P30	NR	<i>p30</i> (D)	NR	21

^a F, frameshift mutation; D, deletion. The frameshift mutation in the *hmw2* gene of the class I-2 mutant results in a 952-amino-acid product of the intact 1,818-amino-acid protein. The frameshift mutation in the *p30* gene of the class II-3 mutant results in a 286-amino-acid product of the intact 274-amino-acid protein. The frameshift mutation in the *hmw1* gene of mutant M6 results in a 589-amino-acid product of the 1,018-amino-acid protein. The deletion in the *p30* gene of M6 results in a 227-amino-acid product of the 274-amino-acid protein. The deletion in the *p30* gene of M7 results in a 203-amino-acid product of the 274-amino-acid protein.

^b +, complementation test was performed.

^c NR, not reported.

revertants before they were inoculated. Transformation of plasmid pKV124 harboring the *p30* gene into the M6 mutant was performed as described previously (11).

Antibodies and immunofluorescence microscopy. Mouse monoclonal antibody M218 against P1 adhesin was provided by P.-C. Hu (12). Rabbit polyclonal antibodies α FP H08-1F, α FP E7B-5, and α FP E7B-17 against HMW1, P90, and P40, respectively, were provided by R. Herrmann (28). A rabbit polyclonal antibody against P30 was provided by G. Layh-Schmitt (21). Mouse polyclonal antibodies against HMW3 and P65 were prepared as described previously by using plasmids H08-3D and F10-2D, respectively (28). Immunofluorescence microscopy was carried out as previously described (34); 200- and 100-fold dilutions of antisera were used as the primary antibodies against HMW3 and P65, respectively. Cell images were obtained with a BX50 microscope (Olympus, Tokyo, Japan) and were digitized by using a Photometrics CoolSNAP charge-coupled device camera (Roper, Atlanta, Ga.).

Thin-section electron microscopy. Thin-section electron microscopy was carried out as previously described, with slight modifications (35). Briefly, 20 ml of mycoplasma cells was collected by centrifugation at $15,000 \times g$ and 4°C for 10 min, fixed with a solution containing 3.0% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline for 60 min at 4°C , washed with 0.1 M sodium phosphate buffer (pH 7.0), and collected by centrifugation. The cell pellet was embedded in 1% low-melting-temperature agarose in 0.1 M sodium phosphate buffer (pH 7.0) and cut into 1-mm-square blocks. Cells in agarose blocks were incubated with 0.1% (wt/vol) osmium tetroxide for 3 h at room temperature. Dehydration was carried out with a series of ethanol washes (70, 80, 90, and 100% ethanol; 10 min each), followed by treatment with propylene oxide for 30 min at room temperature. The agarose blocks were embedded in Quetol 812 resin (OKEN, Tokyo, Japan) according to the manufacturer's protocol and incubated overnight at 37°C and then for 2 days at 65°C . Thin sections were cut

with glass knives and mounted on copper grids. Cells on grids were negatively stained with 2% (wt/vol) uranyl acetate for 3 min and washed with water for 1 min. Specimens were observed with a H7000 transmission electron microscope (Hitachi, Tokyo, Japan). Cell images were digitized with a Dimage multiscan film scanner (Minolta, Tokyo, Japan).

RESULTS

Subcellular localization of cytodherence proteins in cytodherence mutants. We examined the localization of six cytodherence proteins in the wild type and five mutant strains using immunofluorescence microscopy. The characteristics and results obtained for the mutants are summarized in Tables 1 and 2, respectively.

Mutants in class I have frameshift mutations in the *hmw2* gene, resulting in the loss of cytodherence activity (9). Phase-contrast microscopy of the class I-2 mutant cells showed that there was frequent branching (Fig. 1, panels B). Immunofluorescence images of class I-2 stained for HMW1 and HMW3 (Fig. 1, panels B1 and B2) showed fluorescent foci at the tips of branch projections and cell poles with reduced intensities compared to the intensities in the wild-type strain (Fig. 1, panels A1 and A2). The P1 adhesin showed partial localization in the class I-2 mutant (Fig. 1, panel B3), although it was more

TABLE 2. Localization of cytodherence proteins and formation of the electron-dense core in the wild-type and cytodherence-deficient mutant strains^a

Protein or structure	Wild type	Localization or formation in:							
		Class I-2 (<i>hmw2</i>) ^b	Class II-3 (<i>p30</i>)	Class III-4 (<i>p90/p40</i>)	Class IV-22 (<i>p1/p90/p40</i>)	M6-pKV124 (<i>hmw1</i>)	M5 (<i>p90/p40</i>)	M7 (<i>p30</i>)	M6 (<i>hmw1/p30</i>)
HMW1	+ ^c	+/-	+	+	+	Δ	+	+	Δ
HMW3	+	+/-	+	+	+	-	+	+	-
P1	+	(+/-)	+	+	Δ	(-)	+	+	(-)
P90/P40	+	-	+	Δ	Δ	-	Δ	+	-
P30	+	-	Δ	+	+	-	+	Δ	Δ
P65	+	-	+	+	+	-	+	NT	-
Electron-dense core	+	-	+	+	+	NT	+	+	-

^a See Fig. 1 and 2.

^b The genes in parentheses are mutated.

^c + (for proteins), fluorescent foci were observed with intensity comparable to that of the wild-type strain; +/-, fluorescent foci were observed with reduced intensity; Δ , protein was not expressed or was truncated; - (for proteins), no foci were detected; (+/-), signals were partially focused; (-) signals were distributed throughout the cell bodies; NT, not tested; + (for electron-dense core), present in section images; - (for electron-dense core), not present in section images.

FIG. 1

FIG. 1—Continued

FIG. 1—*Continued*

diffuse than in the wild-type strain (Fig. 1, panel A3). The other cytoadherence proteins (P90, P40, P30, and P65) (Fig. 1, panels B4 to B7) showed faint signals that were totally different from those of the wild-type strain (Fig. 1, panels A4 to A7).

The class IV-22 mutant, which lacks expression of P90, P40, and adhesin P1 (4, 17), had a branched cell morphology as determined by phase-contrast microscopy (Fig. 1, panels C). Immunofluorescence microscopy revealed that cytoadherence proteins HMW1, HMW3, P30, and P65 were localized to the short branches (Fig. 1, panels C). In the class IV-22 mutant, fluorescent signals for these cytoadherence proteins were observed in most cells, and their intensities were similar to those in the wild-type strain cells (Fig. 1, panels A and C). No signals were observed for P1, P90, and P40, as expected (data not shown).

Class II mutants have a frameshift mutation in the *p30* gene (33), and the class III-4 mutant lacks expression of P90 and P40 (17). Both classes of mutants had branched cell morphology as determined by phase-contrast microscopy (Fig. 1, panels D and E). Immunofluorescence microscopy revealed that proteins HMW1, HMW3, P1, P90, and P40 were localized to the branch projections in class II-3 (Fig. 1, panels D). In class III-4, proteins HMW1, HMW3, P1, and P30 were also localized to the branches (Fig. 1, panels E).

M6 mutant has two mutated sites in cytoadherence-related genes (20). One of the mutations can be complemented by a plasmid, pKV124, resulting strain M6-pKV124, in which only HMW1 is mutated (11). Phase-contrast microscopy showed the branched cell morphology of this strain, similar to that of the M6 mutant (Fig. 1F), as previously reported (11, 34). Immunofluorescence microscopy showed that P1 was distributed throughout cell bodies in this transformant, and other cytoadherence proteins, including HMW3, P90, P40, P30, and P65, were not localized (Fig. 1, panels F).

Electron-dense core formation in cytoadherence mutants. We examined the electron-dense core in cytoadherence mutants by using thin-section electron microscopy to investigate which proteins are involved in its formation. In the wild-type strain, the cells had an electron-dense core with a terminal button in the attachment organelle (Fig. 2A). In cytoadherence mutants M5, M7, and class IV-22, subcellular structures similar to the electron-dense core in the wild-type strain were observed at cell poles (Fig. 2B to D). We observed similar structures in some other mutants (class II-3 and class III-4) (data not shown), but not in M6 and class I-2 despite examination of more than 50 cell images of each mutant (Fig. 2E and F). These results suggest that HMW1 and HMW2 are essential for the formation of the electron-dense core.

Double staining for cytoadherence proteins in the wild-type and cytoadherence mutant strains. To elucidate the spatial relationships among the cytoadherence proteins, double staining for the P1 adhesin in combination with a second cytoadherence

protein was performed. P90 and P40 colocalized with the P1 adhesin (Fig. 3, panels A4 and A12; also data not shown). The foci for the other cytoadherence proteins appeared to be more punctate than the focus for the P1 adhesin (Fig. 3, panels A1 to A3 and A8 to A11).

Next, we examined the localization of well-focused cytoadherence proteins. Double staining for HMW1 and HMW3 showed that the signals were found at the same position in the wild-type strains (Fig. 3, panels A5 and A13). HMW1 and HMW3 always colocalized in mutants in which they could both be detected, namely, classes I, II, III, and IV (Fig. 3, panels B, C1, D1, and E1) and the M5 and M7 mutants (data not shown). These results show that colocalization of HMW1 and HMW3 was unaffected by the absence of the other cytoadherence proteins (i.e., HMW2, P1, P90, P40, and P30).

We compared the subcellular localization of P30 and P65 with the subcellular localization of HMW3. The signal positions for P30 and P65 did not correspond to those of HMW3 in the wild-type cells, showing that the P30 and P65 proteins are clustered at a position different from the position of HMW1 and HMW3 (Fig. 3, panels A6, A14, A7, and A15). Enlarged cell images showed that signals for P30 and P65 were found more distally than signals for HMW3 in the wild-type strain (Fig. 3, panels A14 and A15). The positional differences between P65 and HMW3 were also observed in class II-3 (Fig. 3, panels C2 and C3). Positional differences between P30 and HMW3 and between P65 and HMW3 were observed more clearly in the class III-3 and class IV-22 mutants (Fig. 3, panels D2 to D5 and E2 to E5).

DISCUSSION

In this study, we examined protein localization in cytoadherence mutants by immunofluorescence microscopy in order to determine the roles of HMW2 and P1 in cytoadherence protein localization. In the class I-2 mutant missing HMW2, localization of HMW1 and HMW3 to the tip of the attachment organelle was found, although the signals were weaker than those in wild-type cells (Fig. 1, panels B1 and B2). Considering that the steady-state levels of the HMW1 and HMW3 proteins in class I mutants are lower than those in the wild-type strain (3, 27), it is unlikely that the localization of these proteins is affected, although HMW2 is essential for stability of these proteins (27). P1 adhesin showed partial focusing, although it was less focused than it was in the wild-type strain. This suggests that HMW2 is not solely essential for focal localization of the P1 adhesin (Fig. 1, panels B). These conclusions are different from those of previous studies in which immunoelectron microscopy was used, in which the authors concluded that HMW1, HMW3, and P1 were not localized in class I mutants (4, 36, 37). This may be due to differences in conditions between the two methods. The other cytoadherence proteins, P90,

FIG. 1. Localization of cytoadherence proteins in wild-type and mutant cells. Cells of the wild type (panels A), class I-2 (panels B), class IV-22 (panels C), class II-3 (panels D), class III-4 (panels E), and M6-pKV124 (panels F) were stained for the cytoadherence proteins HMW1 (panels A1, B1, C1, D1, and E1), HMW3 (panels A2, B2, C2, D2, E2, and F2), P1 (panels A3, B3, C3, D3, E3, and F3), P90 (panels A4, B4, C4, and F4), P40 (panels A5, B5, D5, and F5), P30 (panels A6, B6, C6, E6, and F6), and P65 (panels A7, B7, C7, D7, E7, and F7). The images on the left are immunofluorescence images, and the images on the right are immunofluorescence images merged with corresponding phase-contrast images. Bar, 2 μ m.

FIG. 2. Thin-section electron microscopic images of the wild type (A) and mutants M5 (B), M7 (C), class IV-22 (D), M6 (E), and class I-2 (F). The electron-dense core indicated by arrowheads is attached with a terminal button at the distal end. Bar, 0.3 μ m.

P40, and P30, were not localized in the class I-2 mutant (Fig. 1, panels B), suggesting that these cytoadherence proteins require HMW2 for focal localization or, alternatively, abundant steady-state levels of HMW1, HMW3, or P65.

In the class IV-22 mutant missing the P1, P40, and P90 proteins, HMW1, HMW3, P30, and P65 were localized (Fig. 1, panels C), showing that the P1 adhesin, P40, and P90 are not essential for focal localization of these cytoadherence proteins. In the class II-3 and III-4 mutants, all other cytoadherence proteins except those missing in the mutants were localized (Fig. 1, panels D and E), showing that P30, P90, and P40 are not essential for localization of other cytoadherence proteins.

In a previous study, we demonstrated the failure of cytoadherence proteins to localize in the M6 mutant (34), which expresses truncated HMW1 and P30 proteins (20). Comparing the results with those obtained for the M5 mutant truncated for P30, we concluded that the lack of cytoadherence protein localization was due to the mutation in HMW1. To confirm this conclusion, here we examined protein localization in the M6-pKV124 strain, in which the mutation of P30 is complemented. Signals were not obtained for proteins HMW3, P90, P40, P30, and P65, and the P1 adhesin was distributed throughout the cell bodies (Fig. 1, panels F), which is consistent with the P1 adhesin results obtained by immunoelectron micros-

copy (11). These results were similar to those obtained for the M6 mutant, supporting the assertion that HMW1 is essential for localization of all cytoadherence proteins (34). In class I-2, which lacks the HMW2 protein, the P1 adhesin was less focused than it was in the wild-type strain (Fig. 1, panels A3 and B3), which can be explained by the low steady-state level of the HMW1 protein in class I mutants (3, 27).

The class II-3 and class III-4 mutants are believed to have the same genotypes as the M7 and M5 mutants, respectively, because the class II-3 and M7 mutants express mutated P30 proteins (21, 33) and the class III-4 and M5 mutants do not express P90 and P40 processed from the product of *orf6* encoded in the P1 locus (17, 18). The localization of cytoadherence proteins was examined previously for M7 and M5, but no such examination has been done for the proteins of the class II-3 and class III-4 mutants except for the P1 adhesin in class II-3 (33) and P65 in each of the mutants (13). The present results (Fig. 1, panels D and E) showed that the protein localization in class II-3 and class III-4 is very similar to that in M7 and M5, respectively, supporting our assertion that P30, P90, and P40 do not have an effect on the localization of other cytoadherence proteins.

In a previous study, it was demonstrated that P65 was localized to a rather wide area at the cell protrusions and showed no

FIG. 3. Double staining for cytoadherence proteins in the wild-type and mutant cells. Wild-type cells were stained for P1 and HMW1 (panels A1 and A8), P1 and HMW3 (panel A9), P1 and P30 (panels A2 and A10), P1 and P65 (panels A3 and A11), P1 and P90 (panels A4 and A12), HMW3 and HMW1 (panels A5 and A13), HMW3 and P30 (panels A6 and A14), and HMW3 and P65 (panels A7 and A15). Class I-2 cells were stained for HMW1 and HMW3 (panel B). Class II-4 cells were stained for HMW1 and HMW3 (panel C1) and HMW3 and P65 (panels C2 and C3). Class III-4 (panels D) and class IV-22 (panels E) cells were stained for HMW3 and HMW1 (panels D1 and E1), HMW3 and P30 (panels D2, D4, E2, and E4), and HMW3 and P65 (panels D3, D5, E3, and E5). The first protein number in each pair was stained green, and the second protein number was stained red. Enlarged images are shown in panels A8 to A15, C3, D4, D5, E4, and E5. The images on the left are immunofluorescence images, and the images on the right are immunofluorescence images merged with corresponding phase-contrast images. Bar for enlarged images, 0.4 μ m; bar for other images, 2 μ m.

FIG. 4. Schematic diagram of the spatial arrangement of cytoadherence proteins and the electron-dense core.

signal in the M5 and M7 mutants (34). Jordan et al. (13) showed that the signals for P65 were well focused in the wild-type and class II, III, and IV strains, which is apparently inconsistent with the previous observations, although both antibodies were raised against the product of the same recombinant (13, 28, 34). Here we made polyclonal antibodies from two mice against the product of a P65 recombinant (28, 29) and performed immunofluorescence microscopy. The results obtained with both of the new antibodies showed that P65 signals were well focused in all strains (Fig. 1, panels A7, C7, D7, and E7). We reexamined the M5 mutant by using these new antisera and found that P65 was stained in this strain as well (data not shown). These observations suggest that the disagreement was caused by the difference in the binding affinity or recognized epitopes of the antibodies. The antibody used in the previous work might have recognized the change in conformation or the local amount of P65.

In the doubly stained images, the signal positions of cytoadherence proteins in mutants, including the signal positions of HMW1 and HMW3 in the class I-2 mutant (Fig. 3), for the most part corresponded to each other, although they were not located at cell poles. These observations suggest that the attachment organelles of cytoadherence mutants are trapped in the process of assembly and cannot fully assemble without all of the requisite components present.

We found relatively high background signals when mutant cells were stained for cytoadherence proteins. The higher background signals observed in images of the mutants than in images of the wild-type preparations may have been related to the procedure used for cell adhesion. The positive charge of poly-L-lysine-coated slides may have absorbed small particles in the culture that in turn may have adsorbed antibodies used in our experiments.

All of the mutants which we examined had branched morphology despite their different genetic backgrounds (Fig. 1), suggesting that branched morphology might be caused by the deficiency of cytoadherence. The wild-type's filamentous cell shape with the attachment organelle at one end might be formed by tension generated by cytoadherence and gliding motility.

The thin-section electron microscopy described here showed that HMW1 and HMW2 are essential for formation of the

electron-dense core (Fig. 2). These results are consistent with the observation that both of these proteins can be found in a subcellular fraction that includes the rod structure (32). Proteins P1, P90, P40, and P30 are dispensable for electron-dense core formation, and P1 was partially localized without an electron-dense core in class I-2 (Fig. 1, panel B3), suggesting that these proteins are not components of the electron-dense core. However, these observations cannot eliminate the possibility that these proteins are associated with the electron-dense core.

We examined the spatial relationships of cytoadherence proteins (Fig. 3), and we classified the localization of cytoadherence proteins into three groups, HMW1-HMW3, P1-P90-P40, and P30-P65 (Fig. 4). The proteins in the first two groups are encoded in the same loci on the genome (14, 15), suggesting that there is cooperation among the proteins. Actual physical interactions among P1, P90, and P40 were suggested by chemical cross-linking studies (19, 22). About one-half of P1 molecules are found in the insoluble fraction when the cells are extracted with Triton X-100, but all P1 molecules are solubilized if M5 mutant cells lacking P90 and P40 are treated (18, 21). Considering these observations and the character of P1 adhesin as a transmembrane protein, it is likely that P1 adhesin penetrating the cell membrane is anchored to cytoskeletal structures by P90 and P40. HMW1 was essential for formation of the electron-dense core (Fig. 2). The subcellular localization of HMW1 and HMW3 determined by immunofluorescence analysis may indicate the position of the electron-dense core. If so, the position of P30-P65 may correspond to the terminal button observed in thin sections of the attachment organelle (Fig. 2). P30 is believed to be the second adhesin to P1 (14, 15), and it might be anchored to the terminal button.

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