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Stabilization of Polar Localization of a Chemoreceptor via Its Covalent Modifications and Its Communication with a Different Chemoreceptor

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In the chemotaxis of *Escherichia coli*, polar clustering of the chemoreceptors, the histidine kinase CheA, and the adaptor protein CheW is thought to be involved in signal amplification and adaptation. However, the mechanism that leads to the polar localization of the receptor is still largely unknown. In this study, we examined the effect of receptor covalent modification on the polar localization of the aspartate chemoreceptor Tar fused to green fluorescent protein (GFP). Amidation (and presumably methylation) of Tar-GFP enhanced its own polar localization, although the effect was small. The slight but significant effect of amidation on receptor localization was reinforced by the fact that localization of a noncatalytic mutant version of GFP-CheR that targets to the C-terminal pentapeptide sequence of Tar was similarly facilitated by receptor amidation. Polar localization of the demethylated version of Tar-GFP was also enhanced by increasing levels of the serine chemoreceptor Tsr. The effect of covalent modification on receptor localization by itself may be too small to account for chemotactic adaptation, but receptor modification is suggested to contribute to the molecular assembly of the chemoreceptor/histidine kinase array at a cell pole, presumably by stabilizing the receptor dimer-to-dimer interaction.

Spatial regulation of the subcellular localization of proteins is important for various cellular events in both prokaryotic and eukaryotic cells. In prokaryotes, for example, polar protein localization has been implicated in cell division, virulence, and chemotaxis (24, 31). In the chemotaxis of *Escherichia coli*, a set of transmembrane receptors named chemoreceptors or methyl-accepting chemotaxis proteins (MCPs), together with the histidine kinase CheA and the adaptor CheW, cluster at a cell pole (26, 34). This polar clustering of the chemotactic machinery is thought to be required for normal signal amplification and adaptation (1, 4, 8, 32, 36).

E. coli has four chemoreceptors (Tsr for serine, Tar for aspartate and maltose, Tap for dipeptides, and Trg for ribose and galactose) and one MCP-related protein involved in redox taxis (Aer). A chemoreceptor forms a homodimer regardless of its ligand occupancy state (27). Other chemotaxis signaling proteins (i.e., CheY, which controls the rotational sense of the flagellar motor, and CheZ, which facilitates dephosphorylation of CheY, the methyltransferase CheR, and the methylesterase CheB) also target to the receptor-kinase cluster (2, 5, 33, 34). However, despite growing knowledge of the three-dimensional structures of and interactions between the signaling components, the mechanism that leads to the polar localization of the receptor is still largely unknown.

Receptor methylation, a key process of adaptation, might be a good candidate for a factor affecting the localization of chemoreceptors. First, the formation of an in vitro complex consisting of a cytoplasmic fragment of Tar, CheA, and CheW is facilitated by receptor amidation, which is equivalent to methylation (18, 21). Second, unlike the high-abundance receptors (Tsr and Tar), the low-abundance receptors (Tap and Trg) are not methylated effectively when expressed as the sole chemoreceptors (41) and localize to a cell pole but do not cluster with CheA and CheW (23). Third, recent fluorescence resonance energy transfer (FRET) analyses have demonstrated that receptor methylation decreases sensitivity to an attractant, presumably by cooperative interactions of receptor dimers (35, 36).

Several independent lines of evidence suggest that chemoreceptor dimers interact with each other in vivo. A cytoplasmic fragment of Tsr crystallizes with a unit of a "trimer of dimers" through the interaction at its cytoplasmic *h*ighly *c*onserved *d*omain (HCD) (13, 14), which is conserved among all of the chemoreceptor proteins (17, 42). Based on crystallography, it is proposed that chemoreceptors form a hexagonal network (14, 31). Studies with mutagenesis, cross-linking, and immunoprecipitation showed that Tar and Tsr interact with each other through the HCD in vivo (1). More recently, in vivo experiments with trifunctional cross-linking reagents have strongly suggested that mixed trimers of Tar and Tsr dimers exist in the cell under normal conditions (37). Studies with chemically synthesized multivalent ligands suggested that an attractant response is amplified by the interaction between dimers of the same and different chemoreceptor species (8, 16). It was recently demonstrated by FRET analyses that receptor cooperativity is affected by the expression level of a chemoreceptor and the existence of other chemoreceptors (36).

In this study, we found that the polar localization of Tar fused to green fluorescent protein (GFP) is influenced by its own amidation (methylation) state and the expression of another chemoreceptor (Tsr). Covalent modification is suggested to stabilize polar localization of the chemoreceptors, presum-

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ably through interdimer interactions. The greater tendency of amidated forms of the receptors to localize to the cell pole may be critical in biogenesis of the polar clusters and may provide an explanation for why the receptors are initially translated in the half-amidated forms rather than the nonmodified (all-glutamate) forms.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used in this study are derivative of *E. coli* K-12. Strain RP437 is wild type for chemotaxis (29). Strains HCB339 (39), HCB436 (40), and HCB437 (40) lack all four chemoreceptors (MCPs). In addition, strain HCB436 lacks CheB and CheR and strain HCB437 lacks all of the Che proteins. Strain AW518 lacks Tsr (15).

The vector plasmids pBAD24 (carrying the ampicillin resistance gene [Ap^r]) and pBAD33 (carrying the chloramphenicol resistance gene [Cm^r]) (9) carry the *araBAD* promoter and the *araC* gene, which encodes the positive and negative regulator of the *araBAD* promoter. Plasmid pEGFP, which encodes enhanced GFP, and plasmid pTrcHisB, which carries the *trc* promoter, the $lacI^q$ gene, and the *bla* gene, were purchased from Clontech and Invitrogen, respectively.

The pBAD24-based plasmid pDS223 carries GFP-CheR-D154A (33). The methylation sites of Tar-QEQE on pLC113 plasmids were mutagenized to yield pDS1000 (Tar-EEEE) and pDS1014 (Tar-QQQQ), respectively.

Construction of the plasmids encoding Tar-GFP. The PstI-HindIII fragment of pDS1020, carrying the gene for Tar-QEQE-GFP placed downstream of the *trc* promoter (10), was cloned into the PstI-HindIII sites of pHS401 (H. Sakamoto and I. Kawagishi, unpublished), carrying the gene for Tar placed downstream of the *araBAD* promoter, to yield the plasmid encoding Tar-QEQE-GFP under the control of the *araBAD* promoter (named pDS1030). The NdeI-PvuII fragments of pDS1000 (encoding Tar-EEEE) and pDS1014 (encoding Tar-QQQQ) were cloned between the corresponding sites of pDS1030 to yield the plasmids encoding Tar-EEEE-GFP (named pDS1031) and Tar-QQQQ-GFP (named pDS1032), respectively. The PstI-HindIII fragments of pDS1031 and pDS1032 were cloned into the corresponding region of pDS1020 to yield the plasmids encoding Tar-EEEE-GFP (named pDS1021) and Tar-QQQQ-GFP (named pDS1022) under the control of the *trc* promoter, respectively.

Immunoblotting. Receptor methylation and expression levels of proteins were monitored by immunoblotting as described previously (28) with anti-Tsr serum (11), which cross-reacts with Tar, or anti-GFP antibody (Molecular Probes). The first antibodies were detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G (Vector Laboratories) or horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (New England BioLabs).

Fluorescence microscopy. Observations of fluorescence of Tar-GFP or GFP-CheR were carried out essentially as described previously (2, 33). The culture conditions were similar to those used for the immunoblotting analyses. Cells were harvested, washed twice with MLM (10 mM potassium phosphate buffer [pH 7.0], 0.1 mM EDTA, 10 mM DL-lactate, 0.1 mM methionine), and resuspended in MLM medium. In order to examine the effects of Tsr on the localization of Tar-GFP, HCB436 ($CheA^+$ $CheW^+$) cells were transformed with plasmid pDS1021 (encoding Tar-EEEE-GFP) and then further transformed with plasmid pBAD33 or pOH351 (tsr^+; Motohiro Homma and Ikuro Kawagishi, unpublished results). The transformants were grown in TG medium (1% tryptone, 0.5% NaCl, 0.5% [wt/vol] glycerol) supplemented with 50 μg/ml ampicillin and 25 μg/ml chloramphenicol with vigorous shaking at 30°C. After 3 h, various concentrations of arabinose (for expression of Tsr) were added to the cultures. Cells were grown further for 0.5 h, harvested, washed twice with MLM, and resuspended in MLM medium. Small aliquots of the cell suspensions were spotted onto glass slides coated with 0.5% agarose and were observed under an inverted fluorescence microscope IX70 (Olympus). The images were recorded and processed by using cooled charge-coupled-device camera CoolNAP-FX/OL (Roper) and the software Meta Morph version 5.0r4 (Roper).

RESULTS

Construction and characterization of Tar-GFP. To investigate factors which affect the polar localization of the chemoreceptor (MCP), we first tested the effect of methylation states of the aspartate chemoreceptor (Tar) on its own polar localization. Nascent Tar has four potential methylation sites: Q295, E302, Q309, and E491, collectively referred to as QEQE (Fig. 1A). The two glutamine residues (Q) are deamidated by CheB to become methylatable glutamate residues (E). A glutamine residue mimics methylated glutamate residue (Em) (3, 6), and therefore Tar-QQQQ represents the fully methylated form of Tar. To examine the effect of receptor methylation, we therefore introduced the EEEE and QQQQ configurations into Tar-GFP. In the swarm assay using tryptone semisolid agar (0.3%), HCB339 (ΔMCP) cells expressing the Tar-QEQE-GFP protein (10) formed a swarm ring (Fig. 1B). Although the level of functionality cannot be quantified from these plates, HCB339 cells expressing the GFP fusion protein swarmed only slightly slower than those expressing the intact Tar protein (data not shown), indicating that the GFP construct retains essential receptor function. All of the Tar-GFP proteins were expressed in strains HCB436 (ΔMCP ΔCheRB; hereafter referred to as $CheA^+$ $CheW^+$) and HCB437 (ΔMCP ΔCheAWRBZY; hereafter referred to as $CheA^-$ $CheW^-$). Immunoblotting analyses revealed that their expression levels were comparable (Fig. 1C) and did not exceed the total amount of chromosome-encoded Tar and Tsr (data not shown).

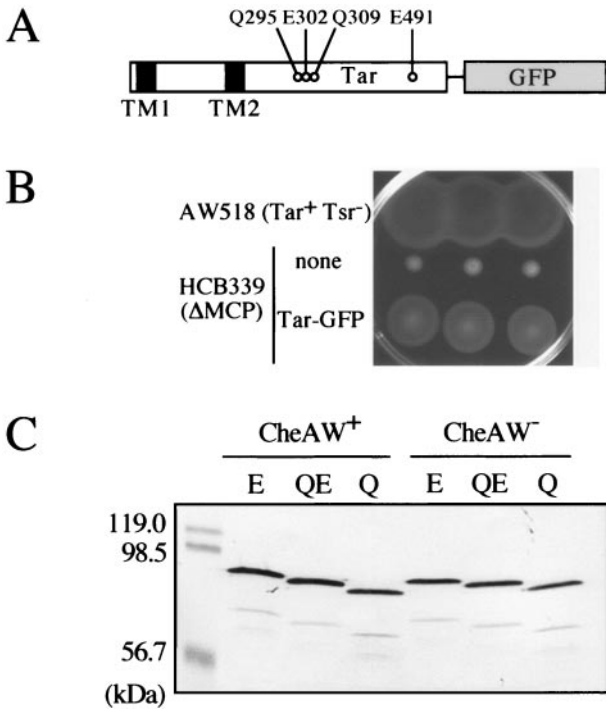

FIG. 1. Construction and characterization of Tar-GFP. (A) Schematic illustration of Tar-GFP. Open circles indicate four methylation sites (Q295, E302, Q309, and E491). Filled and shaded boxes represent two transmembrane domains (TM1 and TM2) of Tar and GFP, respectively. (B) Swarming abilities of cells expressing Tar-QEQE-GFP. AW518 (*tsr*) was used as a control. Shown is the swarming of HCB339 (ΔMCP) cells carrying control vector or the plasmid encoding Tar-QEQE-GFP. Cells were inoculated onto a semisolid agar plate (0.3%) and incubated at 30°C for 12 h. (C) Expression levels of Tar-GFPs in strains HCB436 (ΔMCP $CheA^+$ $CheW^+$) and HCB437 (ΔMCP $CheA^-$ $CheW^-$). E, Tar-EEEE-GFP; QE, Tar-QEQE-GFP; Q, Tar-QQQQ-GFP.

Effect of covalent modification on polar localization of Tar-GFP. Tar-QEQE-GFP localized to cell poles in the presence of

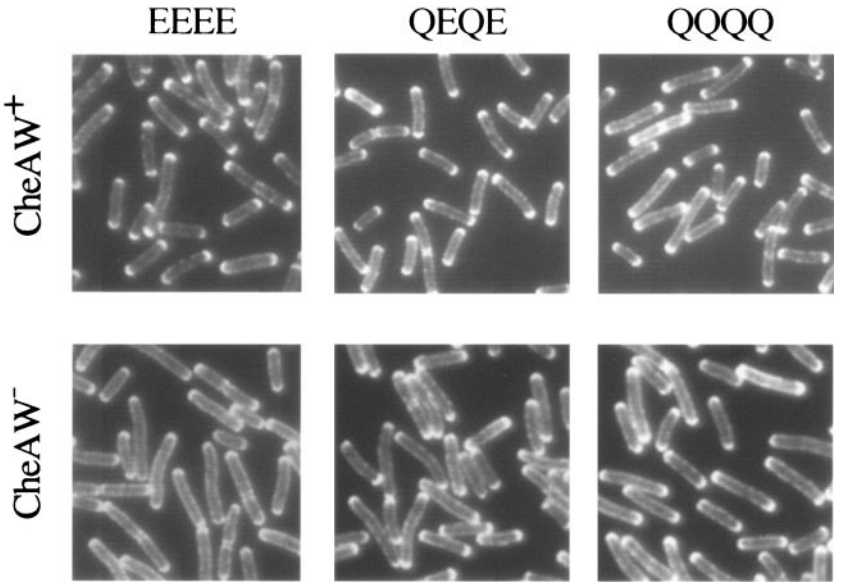

FIG. 2. Effect of covalent modification of localization of Tar-GFP. Shown are the subcellular localizations of the variously amidated versions of Tar-GFP in strains HCB436 (ΔMCP CheA$^+$ CheW$^+$; upper) and HCB437 (ΔMCP CheA$^-$ CheW$^-$; lower). EEEE, Tar-EEEE-GFP (left); QEQE, Tar-QEQE-GFP (middle); QQQQ, Tar-QQQQ-GFP (right).

CheA and CheW, but the extent of its polar localization was reduced substantially in the absence of CheA and CheW (10) (Fig. 2). Microscopic observation detected no obvious difference in the localization among the variously amidated versions of Tar-GFP in the CheA$^+$ CheW$^+$ background (strain HCB436) (Fig. 2). By contrast, in the CheA$^-$ CheW$^-$ background (strain HCB437), Tar-QQQQ-GFP localized much more effectively to cell poles than Tar-EEEE-GFP (Fig. 2).

To quantify the effect of covalent modification on localization, images of cells were recorded, and the fluorescence intensity was quantitated by scanning the image in the direction of the major (length) and minor (width) axes for each cell (Fig. 3A). The maximum intensity of all scans was subsaturating and therefore quantifiable. The difference between the peak and the basal lines was defined as "intensity." Forty HCB437 (CheA$^-$ CheW$^-$) or HCB436 (CheA$^+$ CheW$^+$) cells expressing either the EEEE or QQQQ version of Tar-GFP were examined, and the results are presented in histograms (Fig. 3B). In the CheA$^-$ CheW$^-$ strain, the QQQQ version more effectively localized in the major axis than did the EEEE version. These quantitative results reinforce the qualitative observations made by eye as described above. Even in the CheA$^+$ CheW$^+$ strain, the distribution of major-axis fluorescence intensity of the amidated version was slightly but significantly shifted to the higher values (i.e., to the right in the histogram) compared with that of the demethylated/deamidated one (major axis), suggesting that the methylation (amidation) level of Tar influences its own subcellular localization even in the wild-type background.

Effect of receptor covalent modification on polar localization of GFP-CheR. Because the effect of covalent modification described above was subtle, especially in the presence of CheA and CheW, and localization of Tar-GFP does not exactly reflect that of Tar itself (2), we wanted to examine whether localization of the nonfusion chemoreceptor is also influenced by its own methylation. Since CheR targets to the NWETF sequence (33) and there is no known regulation of this interaction, its localization should reflect that of the chemoreceptor. We therefore examined the effect of receptor methylation on localization of GFP-CheR. In this assay, we used a mutant version (D154A) of GFP-CheR that lacks enzymatic activity to alter the methylation state of the receptor (33); otherwise, the EEEE receptor would have been methylated. We previously showed that the mutation D154A does not impair a polar targeting of GFP-CheR (33). HCB436 cells (CheA$^+$ CheW$^+$) that lack CheR and CheB as well as all four chemoreceptors were transformed first with a plasmid encoding Tar-EEEE (pDS1000) or Tar-QQQQ (pDS1014) and then with a plasmid encoding GFP-CheR-D154A. For each amidation state, more than 500 cells were observed, and the fraction of cells with polar fluorescent spots was scored. The observations were triplicated, and the average values of the polar fraction are shown with standard deviations in Fig. 4. GFP-CheR showed slightly but significantly more polar bias when coexpressed with the QQQQ receptor than with the EEEE receptor (Fig. 4), supporting the notion that receptor methylation affects polar localization of the receptor itself.

Communication between two different chemoreceptors. It has been reported that dimers of the same or different chemoreceptor species interact with each other (1, 8, 16). This prompted us to examine whether the interaction between different chemoreceptor dimers in vivo is also involved in their polar localization. HCB436 (CheA$^+$ CheW$^+$) cells expressing Tar-EEEE-GFP were transformed with plasmid pBAD33 (vector) or pOH351 (pBAD33-*tsr*). Immunoblotting analyses showed that increasing concentrations of arabinose resulted in increasing levels of Tsr but not of Tar-EEEE-GFP (Fig. 5). We observed subcellular localization of Tar-EEEE-GFP in the presence of various concentrations of arabinose (Fig. 6A and B). As the expression level of Tsr increased, the extent of polar

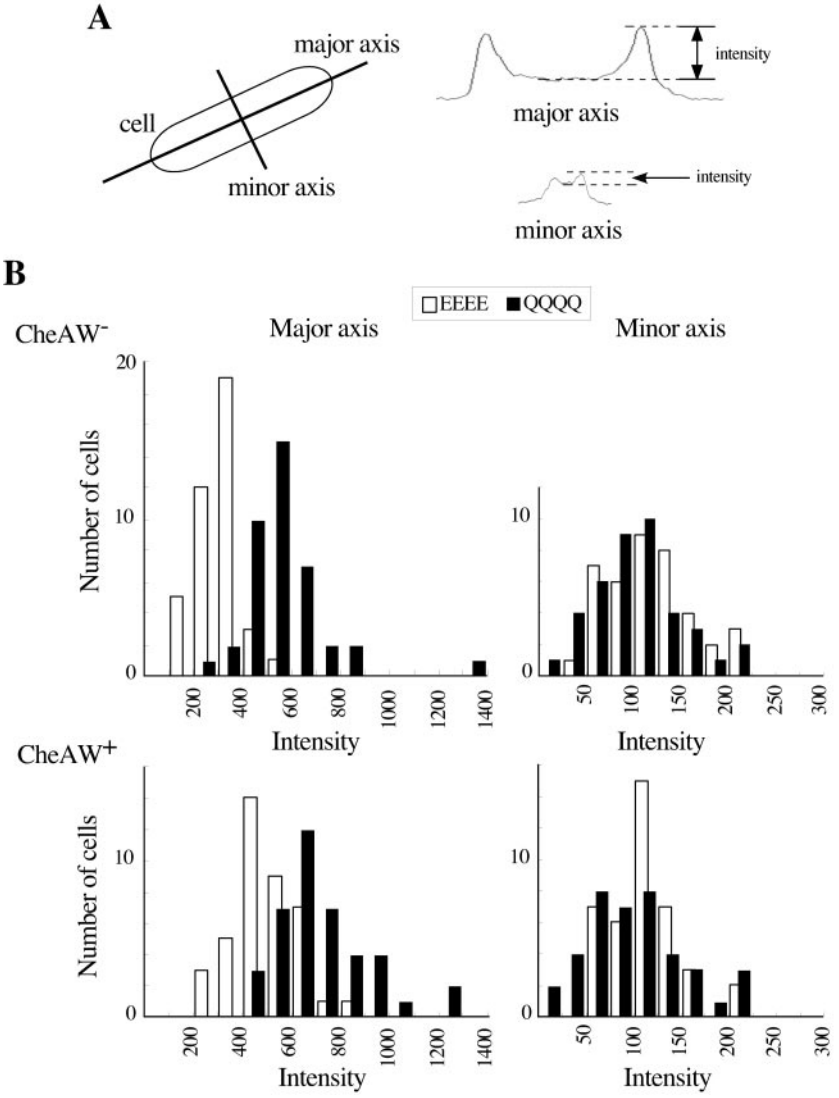

FIG. 3. Quantitative analysis of the effect of covalent modification on receptor localization. (A) Schematic illustration of the quantification procedure. Each cell was scanned for fluorescence intensity in its major and minor axes with the line scan mode of the image-analyzing software (MetaMorph version 5.0r4). The "fluorescence intensity" of a cell in one axis was defined as the difference between the highest and the lowest values (in arbitrary units) within the cell, as indicated in the diagram. (B) Histograms of fluorescence intensities of 40 HCB437 (ΔMCP CheA$^-$ CheW$^-$; upper) or HCB436 (ΔMCP CheA$^+$ CheW$^+$; lower) cells expressing the EEEE (open bars) or QQQQ (filled bars) version of Tar-GFP in major (left) and minor (right) axes. Three independent experiments were carried out, and representative data are shown.

localization of Tar-EEEE-GFP appeared to increase. We carried out semiquantitative analysis of this effect (Fig. 6C). Observed cells were divided into three classes in terms of the localization of fluorescence: completely polar, polar and lateral, and nonpolar. With increasing levels of Tsr, the numbers of nonpolar and completely polar cells decreased and increased, respectively. In the presence of 200 μM arabinose, the proportion of completely polar cells reached a maximum, whereas a higher concentration (2 mM) of arabinose decreased the number of completely polar cells and increased the number of polar and lateral cells (see the Discussion). Even in HCB437 (CheA$^-$ CheW$^-$) cells, increasing levels of Tsr promoted polar localization of Tar-EEEE-GFP (Fig. 6B). These results suggest that a chemoreceptor can recruit a different chemoreceptor species to a pole, presumably by coclustering.

DISCUSSION

In this study, we found factors stabilizing the polar localization of the aspartate chemoreceptor (MCP) Tar using its GFP fusion derivative. It was previously shown that the polar localization of chemoreceptors does not require either CheR or CheB (22). However, this does not necessarily mean that covalent modification has no effect on receptor localization. Even in Δ*cheR* and Δ*cheB* strains, chemoreceptors were not fully deamidated and methylated, respectively (data not shown), and therefore these strains might contain chemoreceptors with various methylation/amidation states. Consequently, the difference in methylation levels between the two strains might not be very large. Moreover, when another amidated receptor (Tsr) is coexpressed, Tar-EEEE-GFP could be localized effectively to

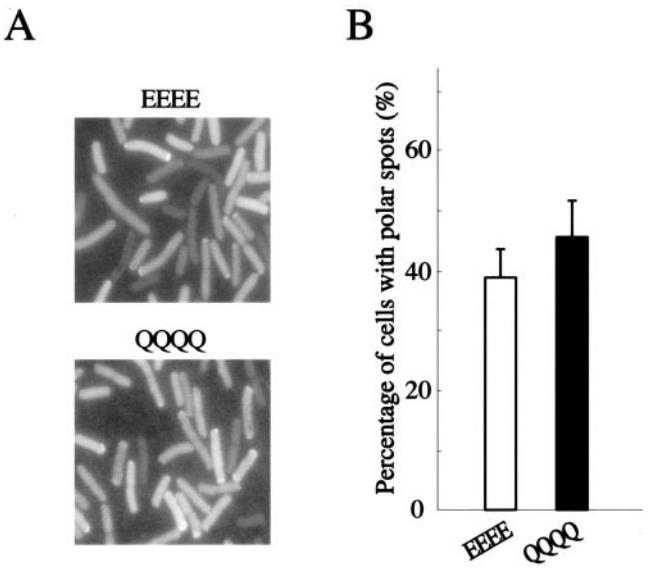

FIG. 4. Effect of receptor methylation on subcellular localization of GFP-CheR that lacks catalytic activity. The GFP-CheR fusion protein with a substitution (D154A) at the catalytic center of CheR was coexpressed with the EEEE and QQQQ forms of Tar in HCB436 (ΔMCP $CheA^+$ $CheW^+$) cells in the presence of 0.5 mM arabinose (for GFP-CheR) and 0.1 μM sodium salicylate (for Tar). (A) Fluorescence images. (B) Fraction of cells with polar fluorescent spots. More than 500 cells were scored for each condition. The observations were triplicated, and the average values of the polar fraction are shown with standard deviations. Note that no polar spot was observed for HCB436 cells expressing GFP-CheR without any receptor (not shown, but see reference 33).

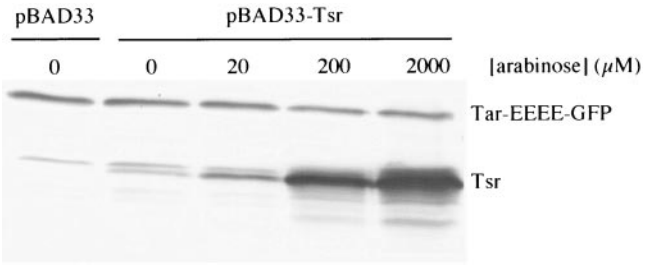

FIG. 5. Coexpression of Tsr with Tar-EEEE-GFP. Expression levels of Tar-EEEE-GFP and Tsr in HCB436 (ΔMCP $CheA^+$ $CheW^+$) cells were detected with anti-Tsr, which cross-reacts with Tar. To induce the expression of Tsr, arabinose was added to cell culture at the indicated concentrations. The same cultures were used in microscopic observation (Fig. 6).

cell poles, as shown in Fig. 6. A more recent immunoelectron microscopic study by Lybarger et al. (25) showed that localization of low-abundance chemoreceptors, but not high-abundance ones, is facilitated by amidation. Their results are partially inconsistent with ours. We found that amidation slightly but significantly enhances localization of the GFP fusion to the high-abundance chemoreceptor (i.e., Tar). This does not seem to be an artifact, because the localization of GFP-CheR, which targets to the high-abundance chemoreceptors, was also enhanced by amidation of nonfusion Tar, presumably reflecting the change in the localization of Tar. In fact, Liberman et al. (20) also detected a slight decrease in localization of yellow fluorescent protein fusions to CheA and CheZ coexpressed with the EEEE version of Tar compared to those coexpressed with the QEQE version. We suspect that fluorescence microscopy could pick up more subtle changes in localization than immunoelectron microscopy because the effect of covalent modification on polar localization was subtle, especially in the $CheA^+$ $CheW^+$ strain. In the $CheA^-$ $CheW^-$ strain, the difference was greater, suggesting that CheA and CheW stabilize the polar cluster containing less-methylated forms of chemoreceptors. It should be noted that HCB437, which was used as a $CheA^-$ $CheW^-$ strain, is missing CheZ and CheY as well as the MCPs CheA, CheW, CheR, and CheB. We cannot rule out the possibility that CheZ and CheY affect the receptor localization in strain HCB436. However, it is unlikely, since the expression of CheA and CheW can readily reverse a decrease in the interdimer cross-linking of Tar in a stain lacking all of the Che proteins and MCPs, which reflects decreased polar localization of Tar (10). It should also be noted that fluorescence microscopy cannot unambiguously distinguish between polar localization and polar clustering.

An amidated cytoplasmic fragment of Tar effectively forms a complex with CheA and CheW in vitro, whereas the deamidation of the fragment results in dissociation of the complex (18, 19), suggesting that electrostatic repulsion between deamidated/demethylated methylation helices might cause the polar cluster to be unstable. However, receptor modification states do not affect in vivo cross-linking efficiencies at the trimer contact interface within the cytoplasmic domain (37). In contrast, our preliminary results suggest that cross-linking of Tar in the periplasmic domain is sensitive to its modification state (Hiroki Irieda, Motohiro Homma, and Ikuro Kawagishi, unpublished results). It is therefore reasonable to assume that covalent modification of a chemoreceptor may stabilize the interaction between trimers of its dimers and/or may alter dynamics of dimers within a trimer unit that has not been observed in cytoplasmic cross-linking assays.

It is still unclear how chemoreceptors localize to a cell pole. Tar-GFP, after induced, seems to appear first at lateral regions of the membrane and later localized at a pole (D. Shiomi, M. Yoshimoto, M. Homma, and I. Kawagishi, submitted for publication). Whatever the mechanism of migration, once Tar arrives at a cell pole, it has to be captured there, as proposed for SpoIVFB of *Bacillus subtilis* (30). This capture mechanism might involve, if not strictly require, receptor clustering, since a deletion of the cytoplasmic domain named HCD, which is required for the interactions with neighboring dimers (to form the "trimer of dimers" structure) and with CheA and CheW (to form the ternary complex), abolishes polar localization of Tar-GFP (D. Shiomi and I. Kawagishi, unpublished results). Receptor methylation and interdimer interactions may play a role in this step. The two Gln residues (positions 295 and 309) of Tar are posttranslationally deamidated by CheB to become methylatable Glu residues (12). Our finding that receptor amidation influences polar localization of the receptor-kinase complex might explain why some of the methylation sites are encoded as Gln rather than Glu. The nascent translational product of Tar (QEQE) is more apt to form a polar complex with CheA and CheW than the fully deamidated form (EEEE). If most of the CheB, CheA, and CheW molecules

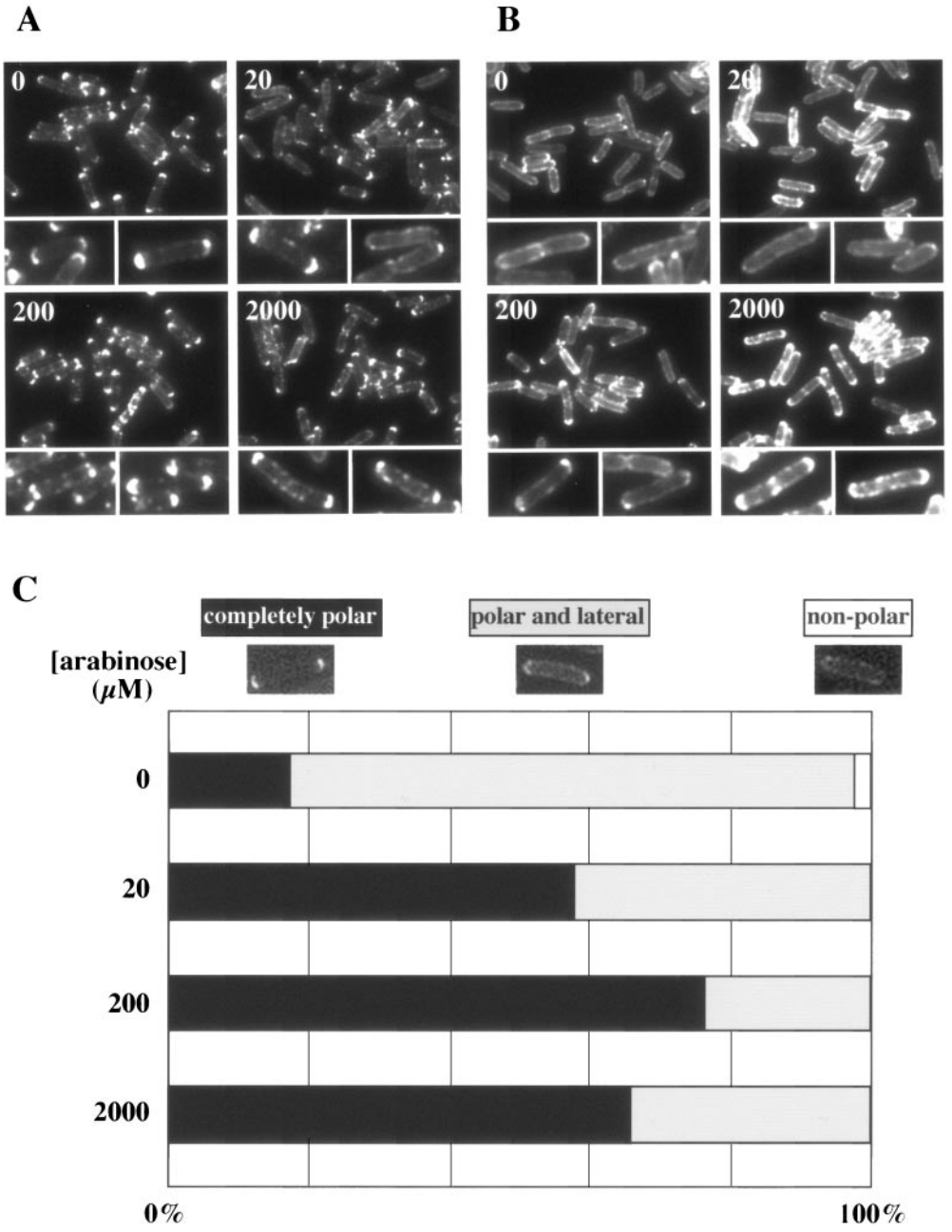

FIG. 6. Effect of Tsr on subcellular localization of Tar-EEEE-GFP. (A and B) Fluorescence micrographs of HCB436 (ΔMCP CheA$^+$ CheW$^+$; panel A) and HCB437 (ΔMCP CheA$^-$ CheW$^-$; panel B) cells expressing Tar-EEEE-GFP with various levels of Tsr. Arabinose was added at the indicated concentrations to induce the expression of Tsr. The same cultures were used in immunoblotting (Fig. 5). (C) Semiquantitative characterization of the effect of Tsr on subcellular localization of Tar-EEEE-GFP in strain HCB436 (ΔMCP CheA$^+$ CheW$^+$). Fluorescent cells were divided into three categories in terms of polar localization (completely polar, polar and lateral, and nonpolar). The bar graph shows the percentages of cells belonging to these categories (filled, shaded, and open bars, respectively), with the representative micrographs above it. Three independent experiments were carried out, and representative data are shown.

localize to the polar clusters, then chances are that a receptor will remain in the QEQE state, and therefore in a polarly targeted trimer of dimers, until it reaches the pole. Once incorporated into the polar receptor array, the deamidation of the methylation sites would not remove Tar from the cluster consisting of variously methylated/amidated forms of Tar and other chemoreceptors as well as CheA and CheW, which stabilize the polar cluster.

The coexpression of Tsr enhanced localization of Tar-GFP to cell poles, suggesting that interaction among chemoreceptors contributes to polar localization. Consistent with this interpretation, several lines of evidence suggest directly or indi-

rectly that different species of chemoreceptors can interact with each other and cluster together (1, 8, 13, 16, 32). A low-abundance receptor (Trg or Tap) is poorly methylated when expressed as a sole chemoreceptor, but its methylation is enhanced by the coexpression of a high-abundance receptor (Tsr or Tar) (7, 38, 42). It was shown that Trg or Tap, when expressed as a sole chemoreceptor, localizes to a cell pole but does not cluster (23). Gestwicki and Kiessling (8) and Lamanna et al. (16) showed that multivalent ligands, which bind to Trg, stabilized the receptor-kinase cluster, and treatment of cells with the multivalent ligands caused amplification of the response to serine. Indeed, localization of low-abundance receptors is more sensitive to the modification state than that of high-abundance receptors (25). In vivo cross-linking studies (1, 37; Irieda et al., unpublished) showed that different types of chemoreceptors interact with each other through their HCD. Such a polar cluster consisting of different chemoreceptors might be required for signal amplification and the stable polar localization of low-abundance and/or demethylated chemoreceptors. There is a possibility that the recruitment of Tar to cell poles by Tsr is not a Tsr-specific event. Rather, the effect might result from "displacement" from the lateral cytoplasmic membrane because of overexpression of any membrane protein. This possibility can be tested by examining whether the transmembrane osmosensor histidine kinase EnvZ, a GFP fusion of which is distributed throughout the cytoplasmic membrane (D. Shiomi and I. Kawagishi, unpublished results), or other membrane proteins are able to promote polar localization of the Tar-EEEE protein.

Although Tar-GFP localized to cell poles more effectively with increasing expression levels of Tsr, the number of completely polar cells decreased significantly when Tsr was overexpressed in the presence of 2 mM arabinose. We also observed that Tar-GFP, when overproduced, localized not only to cell poles but also to lateral cytoplasmic membranes (data not shown). At least some of these lateral populations of chemoreceptors are thought to be associated with or localized in the vicinity of the Sec protein translocation complexes (D. Shiomi, M. Yoshimoto, M. Homma, and I. Kawagishi, submitted for publication). The amounts of CheA and CheW could be limiting, and/or there might be no space at cell poles for too many or too-large clusters. The in vivo molar ratio of receptor, CheA, and CheW in a polar cluster has not been determined precisely. It is possible that the ratio may vary under different physiological conditions, which might be related to a variation in cooperativity that has been demonstrated by recent FRET analyses (36).

In brief, the results obtained in this study provide snapshots of a dynamic process of protein-protein interactions in the chemotactic signaling system. Understanding this system in detail will require the development of probes that can monitor changes in localization during the time course of excitation and adaptation elicited by a chemotactic stimulus.

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