Published in final edited form as:

Mol Microbiol. 2011 February ; 79(3): 677–685. doi:10.1111/j.1365-2958.2010.07478.x.

Chemotaxis Kinase CheA is Activated by Three Neighboring Chemoreceptor Dimers as Effectively as by Receptor Clusters

Mingshan Li¹, Cezar M. Khursigara^{2,†}, Sriram Subramaniam², and Gerald L. Hazelbauer^{1,*}

¹ Department of Biochemistry, 117 Schweitzer Hall, University of Missouri-Columbia, Columbia, MO 65211

² Laboratory of Cell Biology, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

Abstract

Chemoreceptors are central to bacterial chemotaxis. These transmembrane homodimers form trimers of dimers. Trimers form clusters of a few to thousands of receptors. A crucial receptor function is 100-fold activation, in signaling complexes, of sensory histidine kinase CheA. Significant activation has been shown to require more than one receptor dimer but the number required for full activation was unknown. We investigated this issue using Nanodiscs, soluble, nanoscale (~10 nm diameter) plugs of lipid bilayer, to limit the number of neighboring receptors contributing to activation. Utilizing size-exclusion chromatography, we separated primary preparations of receptor-containing Nanodiscs, otherwise heterogeneous for number and orientation of inserted receptors, into fractions enriched for specific numbers of dimers per disc. Fractionated, clarified Nanodiscs carrying ~5 dimers per disc were as effective in activating kinase as native membrane vesicles containing many neighboring dimers. At five independently inserted dimers per disc, every disc would have at least three dimers oriented in parallel and thus able act together as they would in native membrane. We conclude full kinase activation involves interaction of CheA with groups of three receptor dimers, presumably as a trimer of dimers, and that more extensive interactions among receptors are not necessary for full kinase activation.

Keywords

Bacterial chemotaxis; bacterial signaling; histidine kinases; protein phosphorylation; signaling complexes

INTRODUCTION

Chemoreceptors are central components in the molecular machinery that mediates bacterial chemotaxis (Hazelbauer *et al.*, 2008). These receptors, most of which are transmembrane proteins (Zhulin, 2001), interact with histidine kinase CheA and the coupling protein CheW to form signaling complexes. In these complexes, the autophosphorylation activity of CheA is enhanced ~ 100-fold and placed under receptor control (Hazelbauer and Lai, 2010). Signaling complexes form clusters in the cytoplasmic membrane that can contain from a few to thousands of receptors (Maddock and Shapiro, 1993; Gestwicki *et al.*, 2000; Wadhams and Armitage, 2004; Zhang *et al.*, 2007; Briegel *et al.*, 2009; Greenfield *et al.*, 2009). Interactions among multiple receptors and kinases in such clusters are thought to be crucial

*Corresponding author: Telephone: (573) 882-4845. Fax: (573) 882-5635. hazelbauer@missouri.edu.

†Current address: Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

for the notable sensitivity and dynamic range of the chemotactic response (Hazelbauer *et al.*, 2008). To what degree does the 100-fold activation of kinase by receptors in signaling complexes involve or require interactions among multiple neighboring chemoreceptors? We investigated this issue using Nanodiscs.

Chemoreceptors

Bacterial chemoreceptors are extended helical coiled-coil proteins for which the fundamental structural unit is a homodimer (Hazelbauer *et al.*, 2008). Homodimers associate as trimers of receptor dimers (Kim *et al.*, 1999; Studdert and Parkinson, 2004), an oligomeric organization that is prevalent if not universal across bacterial diversity (Briegleb *et al.*, 2009). In all bacterial species examined, chemoreceptors cluster in extensive patches (Maddock and Shapiro, 1993; Gestwicki *et al.*, 2000; Wadhams and Armitage, 2004; Zhang *et al.*, 2007; Briegel *et al.*, 2009; Greenfield *et al.*, 2009). The levels of receptor interaction are illustrated in Fig. 1A. Besides forming signaling complexes that enhance and control the activity of the sensory kinase, receptors are central components in sensory adaptation, undergoing covalent modification, methylation and demethylation of specific receptor glutamates. The extent of methylation influences the conformational equilibrium between two forms of the receptor and thus influences the signaling state of the protein (Hazelbauer *et al.*, 2008). Methylation is catalyzed by methyltransferase CheR to form glutamyl methyl esters and demethylation of those esters is catalyzed by methylesterase CheB to recreate a glutamyl side chain. CheB is activated by phosphorylation (Anand *et al.*, 1998). Thus *in vitro* assays are routinely performed with phospho-CheB. In *Escherichia coli*, chemoreceptors such as the aspartate receptor Tar are synthesized with glutamine rather than glutamate at two of four methyl-accepting sites. These glutamines are deamidated by CheB, the same enzyme that catalyzes demethylation, creating glutamates that are then sites of methylation. Glutamines at methyl-accepting sites are in large part functional equivalents of methyl esters (Lai *et al.*, 2006).

Nanodiscs

It has been challenging to address experimentally the functional roles of interactions among chemoreceptors since these transmembrane proteins are fully functional only when intact and inserted into a lipid bilayer and there had been no convenient way to limit interactions among receptor dimers in membranes, whether the membrane be in intact cells, isolated vesicles or the result of reconstitution from membrane components. Nanodiscs provide a way to overcome this experimental challenge (Boldog *et al.*, 2006; 2007). They are soluble, nanoscale (~10 nm diameter) plugs of lipid bilayer surrounded by a belt of amphipathic membrane scaffold protein (Denisov *et al.*, 2004). Transmembrane proteins can be incorporated into Nanodiscs, rendering the proteins water soluble yet maintaining a natural lipid bilayer environment and thus likely a native state (Bayburt and Sligar, 2003). The extent of potential interaction between multiple copies of a transmembrane protein can be reduced or eliminated using Nanodiscs because there is a limited cross-sectional area of lipid bilayer (Denisov *et al.*, 2004) and the average number of inserted membrane proteins can be manipulated by adjusting the preparation ratio of transmembrane protein to scaffold protein (Boldog *et al.*, 2006).

Chemoreceptor Tar from *Escherichia coli* can be efficiently incorporated into Nanodiscs to create water-soluble particles (Fig. 1B) in which the receptor exhibits key activities that are absent in the detergent-solubilized state (Boldog *et al.*, 2006). Tar embedded in native membrane is a substrate for the enzymes of adaptational modification and couples ligand occupancy to the rate of modification through transmembrane signaling. These properties are missing in the detergent-solubilized state but are exhibited by receptors in Nanodiscs (Boldog *et al.*, 2006; 2007). Detailed characterization of methylation rate as a function of

ligand concentration and extent of adaptational modification demonstrated that individual Tar homodimers inserted in Nanodiscs exhibit the full range of ligand- and modification-influenced conformational changes and transmembrane signaling (Amin and Hazelbauer, 2010). Thus interactions among multiple receptors in clusters of signaling complexes are not required or directly involved in conformational and transmembrane signaling within the receptor dimer.

Kinase activation

Tar in Nanodiscs formed functional signaling complexes with CheA and CheW, as documented by kinase activation and dramatic inhibition of the enhanced kinase activity by saturating receptor ligand (Boldog *et al.*, 2006). In contrast to methylation, for which individual receptor dimers were essentially fully functional as reflected in initial rates of modification and control of those rates by ligand and adaptational modification (Amin and Hazelbauer, 2010), Nanodisc preparations with ~1 dimer per disc exhibited almost no activation of kinase. With increasing numbers of receptor dimers per disc, kinase activation increased up to a maximum for primary Nanodisc preparations containing 2.5 to 3 dimers per disc, implying a role for trimers of dimers. However, that maximum was only ~30% of the kinase activation generated by receptors in native membrane (Boldog *et al.*, 2006), where there would be many potentially interacting receptors. It was not clear whether the lower activation by Nanodisc-isolated receptor dimers reflected an inherent limitation of the small number of neighboring dimers in a Nanodisc or some other parameter(s). We now report that upon fractionation and clarification, Nanodiscs containing only a few receptor dimers are as effective at activating kinase as the many neighboring receptors in native membrane.

RESULTS

Insertion of chemoreceptors into Nanodiscs

Nanodisc-embedded chemoreceptors were prepared by removing detergent from mixtures of detergent-solubilized receptor, detergent-solubilized lipid and membrane scaffold protein. A chelated nickel column was used to separate Nanodiscs containing six-histidine-tagged receptors from empty Nanodiscs and other material (Boldog *et al.*, 2007). The average number of receptor dimers per Nanodisc in these primary preparations is a function of the molar ratio of receptor to scaffold protein in the preparation mixture (Boldog *et al.*, 2006). For instance, a five- to ten-fold excess of scaffold protein over chemoreceptor Tar results in receptor-containing Nanodiscs with ~1 receptor dimer per disc whereas a 1:1 preparation ratio of scaffold protein to Tar produces receptor-containing Nanodiscs with an average of ~3 receptor dimers per disc. The number of receptor dimers per disc appeared to be a roughly continuous function of the preparation ratio (Boldog *et al.*, 2006), implying that individual chemoreceptor dimers incorporate independently and randomly into Nanodiscs. Thus a preparation with an average of three receptor dimers per disc includes discs with fewer than three dimers as well as discs with more, presumably in all possible combinations of parallel and anti-parallel receptor orientations (Fig. 1C).

Fractionation of chemoreceptor-containing Nanodiscs

Because this heterogeneity should create heterogeneity in size and shape among receptor-containing Nanodiscs, we submitted primary preparations of Nanodisc-inserted Tar to fractionation by size-exclusion chromatography and determined amounts of receptor and membrane scaffold protein (MSP) in each fraction. Fig. 2 shows data for a representative fractionation, specifically of a preparation averaging 2.7 dimers per disc. Both receptor and scaffold protein were distributed across the elution volume. The broad distribution was consistent with the expected heterogeneity in number and orientation of receptors inserted in

Nanodiscs. Ratios of Tar to scaffold protein were highest in the earliest eluting fractions, decreasing across the elution volume, as would be expected for fractionation from the largest operational size to the smallest. To analyze receptor activities, each fraction was concentrated and clarified by centrifugation. We found that a portion of Tar in each fraction was lost by these manipulations, from <10% for the latest eluting fractions, those expected to contain discs with fewer inserted dimers, to ~90% for the earliest eluting fractions, those expected to contain discs with more dimers. We deduce that some Tar in the primary preparation was not native protein appropriately inserted into Nanodiscs but instead was structurally disrupted and/or ineffectively inserted in the lipid bilayer. Such receptors might form small, partially soluble aggregates or remain soluble in the course of initial preparation of receptor-containing Nanodiscs by interaction with correctly folded, Nanodisc-inserted receptors. After removal of this non-native material, quantification of Tar and scaffold protein in each fraction revealed values for receptor dimers per disc from one to approximately five receptor dimers per disc. This maximum is near the capacity of the cross-sectional area of the Nanodisc lipid bilayer for accommodating inserted receptor dimers (Boldog *et al.*, 2006).

Characterization of fractionated, Nanodisc-inserted chemoreceptors: native structure

Fractions from column separations like those illustrated in Fig. 2 were sources of water-soluble, Nanodisc-embedded Tar substantially enriched for narrower distributions of the number of dimers per disc than primary Nanodisc preparations, as well as being separated from receptor that did not remain soluble during the manipulations. Tar in each fraction was tested for extent of reaction with the two enzymes of adaptational modification in conditions (high enzyme concentration and long incubation times) that resulted in a plateau value for receptor modification. The resulting values for maximal percent of receptors modified are empirical measures of the proportion of Tar with sufficiently native structure to be recognized by the respective enzymes. Notably, Tar in detergent micelles exhibits essentially no modification by CheR or CheB, indicating perturbed structure (Boldog *et al.*, 2006). Fig. 3A shows that by the criteria of deamidation by phospho-CheB and methylation by CheR, a substantial majority of Nanodisc-inserted Tar was in a native state. Slightly more receptor was modified by phospho-CheB than by CheR, a relationship we observe routinely for receptors in native or Nanodisc membrane and which is likely to reflect specific features of the two reactions. As the number of receptor dimers per disc increased, the proportion of modified receptors gradually decreased. This decrease could be the result of reduced accessibility of receptor dimers to the enzymes because of interfering neighbors in the same Nanodiscs, a greater chance of disruption of receptor structure in the process of Nanodisc formation as the ratio of scaffold protein to transmembrane protein decreased or a combination of factors. Consistent with the notion that concentration and clarification of fractions from the size-exclusion chromatography enriched for native receptor by removing non-native Tar, the proportion of Tar in a native state as judged by ability to be modified was 15% to 50% greater at each respective value for dimers per disc than unfractionated primary preparations of Nanodisc-embedded Tar exhibiting the same apparent number of dimers/disc (Fig. 3B and 3C).

Characterization of fractionated, Nanodisc-inserted chemoreceptors: kinase activation

In striking contrast to the pattern for receptor modification, kinase activation as a function of dimers per Nanodisc exhibited a sigmoidal relationship (Fig. 4A). Addition of CheA and CheW to Nanodisc-embedded Tar fractionated on size-exclusion columns like the one illustrated in Fig. 2 resulted in low activation by fractions averaging less than two receptor dimers per disc but rose as a function of the number of dimers per disc to a maximum at 4 to 5 dimers per disc (Fig. 4A). At this maximum, kinase activation by Nanodisc-embedded Tar was equivalent to activation by Tar embedded in native membrane vesicles assayed in the

same conditions. Thus a limited number of neighboring receptor dimers activated kinase as effectively as receptor clusters in native membrane. This activation represented physiologically relevant coupling of receptor and kinase because addition of a saturating concentration of the Tar ligand aspartate eliminated kinase activation (Fig. 4A), indicating functional coupling of ligand binding at one end of the Nanodisc-embedded receptor to the kinase in complex at its opposite end.

It is informative to compare the sigmoidal relationship shown in Fig. 4A with the previous characterization (Boldog *et al.*, 2006) of kinase activation by primary preparations of Nanodisc-embedded Tar (Fig. 4B). For values up to 2.5–3 dimers per disc, kinase activation as a function of dimers per disc was similar for primary and column-purified preparations of Nanodisc-inserted Tar. At higher ratios, activation by primary preparations declined sharply. We attribute that decline to the increasing proportion of non-native receptors in primary Nanodisc preparations at higher numbers of dimers per disc (see above). This pattern would result in overestimates of the number of receptors per disc and would likely interfere with the activation of kinase by dimers in the same Nanodisc that were native.

In the experiments illustrated in Fig. 4A we varied a single parameter, the source of Tar, holding all other parameters constant, including the relative amounts of CheA, CheW and Tar mixed to form signalling complexes. The ratios among those components were determined by optimizing kinase activation by Tar in Nanodiscs (see Experimental procedures). These ratios differed from those we had used in previous studies of kinase activation by Tar in native membrane (Barnakov *et al.*, 1998; Amin and Hazelbauer, 2010) or in our initial characterization of activation by Tar in Nanodiscs (Boldog *et al.*, 2006). Nanodisc-optimized ratios provided 2.5-fold more Tar relative to CheA and approximately one-third the amount of CheW relative to CheA. Although these differences were within the range of ratios used in other studies of kinase activation (Borkovich and Simon, 1991; Ninfa *et al.*, 1991; Chervitz *et al.* 1995; Morrison and Parkinson, 1997; Bornhorst and Falke, 2000; Li and Weis, 2000; Levit and Stock, 2002; Draheim *et al.*, 2005; Lai *et al.*, 2005; Bhatnagar *et al.*, 2010), it was important to investigate whether the ability of only a few Tar dimers in Nanodiscs to activate kinase as effectively as many dimers in native membrane was limited to a particular way of preparing signalling complexes made with Tar from the two sources. Fortunately, we found that kinase activation by Nanodiscs containing ~5 dimers per disc was at least as effective as activation by Tar in native membrane over a range of preparation ratios. Varying the ratio of CheW to CheA used to form signalling complexes over a six-fold range that included the ratio used for Fig. 4A and the ratio we used previously for studies of Tar in native membrane (Barnakov *et al.*, 1998; Boldog *et al.*, 2006; Amin and Hazelbauer, 2010) while holding the CheA: Tar ratio constant did not alter the extent of kinase activation by Tar in Nanodiscs or by Tar in native membrane. Varying the ratio of Tar to CheA used to form signalling complexes at a constant CheA:CheW ratio (Fig. 5) did alter the extent of kinase activation, but at all ratios kinase activation by purified and clarified Nanodisc-embedded Tar at ~5 dimers/disc was at least as great as activation by the same amount of receptor embedded in native membrane. At Tar:CheA $\geq 40:1$, kinase activation by receptors in Nanodiscs reached a plateau, consistent with activation of all available CheA, whereas kinase activation by Tar in native membranes decreased, likely reflecting the negative effects of increasing amounts of bulk native membrane. In any case, the data in Fig. 5 strongly indicate that a limited number of neighboring receptor dimers can activate kinase at least as effectively as many neighboring receptor dimers in native membrane.

Kinase activation by Nanodisc-embedded Tar at ~ 1 dimer per disc

As seen in Fig. 4A, even at ~1 dimer per disc, enriched and clarified preparations of Tar-containing Nanodiscs mediated kinase activation (14 % of maximal) and this activity was

controlled by ligand occupancy. What was the origin of this activation? Could fractionated Nanodisc preparations with average contents just over 1 dimer per disc contain a sufficient number of discs with three dimers to mediate activation? This could be possible if dimer insertions into Nanodiscs were not entirely independent but instead some dimers inserted in parallel as groups of three. To assess directly the stoichiometry of receptors in fractionated Nanodiscs averaging ~1 dimer per disc, we performed electron microscopy of negatively stained specimens. Examination of a fractionated and clarified Nanodisc preparation at ~1.1 Tar dimers per disc, revealed particles with shapes and dimensions expected for Nanodiscs, many of which were associated with needle-like structures (Fig. 6). These needles had the length of receptor dimers, 301 ± 14 Å compared with values of 310 ± 12 Å and 300 ± 11 Å observed in negative-stained images of receptor in native membrane (Weis *et al.*, 2003). We conclude that the needles represented Tar dimers inserted into Nanodiscs. Since Nanodiscs were adsorbed in many orientations on the carbon film, only some were oriented in a side view in which inserted receptors were visible. Nevertheless, we could analyze Nanodiscs with visible receptors to estimate the proportion of discs containing one or more dimers per disc. Of 163 Nanodiscs with visible inserted chemoreceptors, 154 (94.5%) had one dimer, 8 (4.9%) had two and 1 (0.6%) had three. Thus the ability of such a preparation to mediate ~15% of maximal kinase activation could not be explained simply by the presence of individual discs with three parallel dimers. Instead, the significant activation could reflect Nanodisc-isolated receptor dimers interacting individually or in groups with CheA and CheW. These possibilities are being investigated.

DISCUSSION

Deducing the number of receptor dimers required for maximal kinase activation

Fractionated and clarified Nanodiscs carrying approximately five Tar dimers per disc were as effective in activating kinase as native membrane vesicles containing many neighboring Tar dimers (Fig. 4A). Effective activation by this small number of isolated receptor dimers was not a function of a particular ratio in which receptors, CheA and CheW were mixed to form signaling complexes (see Results and Fig. 5). Thus we conclude that the extensive activation of kinase by receptors with many potentially interacting neighbors *in vivo* or *in vitro* reflects concerted action of only a few neighboring receptor dimers.

The specific number of interacting receptor dimers required for maximal kinase activation can be deduced from the relationship between the extent of kinase activation and the number of dimers per Nanodisc (Fig. 4A). Purified Nanodisc preparations with one to two dimers per disc mediated little kinase activation but as dimers per disc increased the extent of activation rose steeply to a maximum as the number approached five. Chemoreceptor dimers are incorporated into Nanodiscs independently (Boldog *et al.*, 2006;2007). At five dimers per disc, every Nanodisc would have at least three parallel receptor dimers, i.e. dimers oriented in the same direction. These three could act together as they would in native membrane, presumably as a trimer of dimers. Had kinase activation by receptors involved groups of more than three physiologically interacting and thus parallel receptors, activation would not have reached a plateau at five dimers per disc, since not all Nanodiscs would contain the required number of parallel receptors. Taken together, our observations indicate that the unit of kinase activation is the receptor trimer of dimers. Thus interaction among the many neighboring receptors in a cluster is not required for effective kinase activation.

Stoichiometry

Our results identify trimers of dimers as the fundamental receptor unit required for kinase activation, but they do not distinguish among alternative ways in which such trimers might interact with kinase CheA, ways that would result in different stoichiometries for the

underlying unit of kinase activation in signaling complexes. Each CheA homodimer (Surette *et al.*, 1996) could bind a receptor trimer of dimers and thus be activated. Alternatively, each subunit of the kinase dimer could bind a receptor trimer of dimers. Recent observations and deductions are consistent with the latter alternative (Khursigara *et al.*, 2008; Erbse and Falke, 2009; Bhatnagar *et al.*, 2010). The ability to isolate individual trimers of dimers in Nanodiscs provides a way to investigate this issue of stoichiometry.

Units of kinase activation and control are different

Chemoreceptors not only activate the kinase but also control its activity as a function of ligand occupancy and adaptational modification. This control is exerted by coupled units of receptor dimers of variable size that can be as large as 10–15 receptor dimers (Li and Weis, 2000; Bornhorst and Falke, 2001; Mello *et al.*, 2004; Sourjik and Berg, 2004; Kentner *et al.*, 2006; Skoge *et al.*, 2006; Endres *et al.*, 2008). This size is significantly larger than the three receptor dimers we found sufficient for maximal kinase activation. This indicates that the receptor unit of kinase activation and of kinase control are distinct. Furthermore, it introduces the possibility that the interactions and mechanisms involved in kinase control are not necessarily the same as those involved in kinase activation. Conversely, effective kinase activation by as few as three receptor dimers indicates that the many small clusters of receptors/signalling complexes that occur across the cytoplasmic membrane (Greenfield *et al.*, 2009) would contribute proportionally to overall kinase activity, but the extent of cooperative control of that contributed activity could be limited. The combination of a distinction between the unit of kinase activation and kinase control with the potential for graded extents of cooperative control identifies previously unappreciated ways in which the chemosensory response could be tuned.

Experimental procedures

Proteins and Nanodiscs

Tar with a six-histidine tag on its carboxyl terminus (Tar-6H), membrane scaffold protein MSP1D1E3(–) (Denisov *et al.*, 2004), hereafter simply “MSP”, and Nanodiscs containing Tar-6H were prepared essentially as described (Boldog *et al.*, 2007) except that samples of dried *E. coli* polar lipids were solubilized by placing in the tube of dried lipid a volume of 50 mM Tris-HCl (pH 7.5), 100 mM cholate equivalent to the volume of the lipid solution before drying, and submitting the tube to gentle agitation with a vortex mixer approximately every 15 min until the solution cleared (~ 4 hours).

Size-exclusion chromatography

Receptor-containing Nanodiscs were fractionated at room temperature (~24°C) with a Tosoh Haas TSK G5000 PW_{XL} 7.8-mm inner diameter × 30 cm column equilibrated in 50 mM Tris-HCl (pH 7.5), 10% wt/vol glycerol, 100 mM NaCl, 0.5 mM EDTA, hereafter disc buffer, and run in the same buffer at 0.4 ml/min, collecting 0.3-ml fractions. Tar and MSP in each fraction were quantified by SDS polyacrylamide gel electrophoresis, Coomassie Brilliant Blue staining, and comparison to standards, for the respective proteins, run on the same gel. Fractions were concentrated in a Amicon Ultra 4 concentrator (Millipore) to > 30 μM Tar, clarified by centrifugation for 2 min at maximal speed (16,100 × g) in a table top Eppendorf Centrifuge 5415D, quick frozen in liquid nitrogen, stored at –80°C, and Tar and MSP quantified as above.

Assays for native receptors: deamidation and methylation

Nanodisc-embedded chemoreceptors were assayed for the proportion of molecules sufficiently native to be recognized and thus deamidated by phospho-CheB by mixing equal

volumes of Nanodisc-embedded Tar and the enzyme, and incubating for 2 h at final concentrations of ≤ 5 μM Tar, 5 μM CheB and 50 mM phosphoramidate in disc buffer containing 10 mM KCl and 10 mM MgCl_2 , or sufficiently native to be recognized and thus methylated by CheR by mixing equal volumes of Nanodisc-embedded Tar and the enzyme in an enriched lysate plus S-adenosylmethionine, and incubating for 2 h at final concentration of ≤ 5 μM Tar, 5 μM CheR and 80 mM S-adenosylmethionine in disc buffer. The proportion of the receptor population modified by either enzyme was determined by SDS-polyacrylamide gel electrophoresis and quantification of bands corresponding to modified and unmodified Tar (Boldog *et al.*, 2006).

Kinase activity

Receptor-stimulated kinase activity was assayed essentially as described (Barnakov *et al.*, 1998). Relative amounts of Nanodisc-embedded receptors, CheA and CheW required to produce maximal kinase activation were optimized by varying individual components, one at a time, through two cycles of optimization. The optimized ratios, 20 Tar: 1 CheA: 5 CheW, resulted in a $\sim 50\%$ increase in the extent of kinase activation by unfractionated Nanodisc-embedded Tar relative to activation by Tar in native membrane (Boldog *et al.*, 2006) but differed from the ratios optimized for kinase activation by receptor in native membrane (6.7 Tar: 1 CheA: 16 CheW). For all ratios used, the components, in disc buffer containing KCl and MgCl_2 , were mixed to yield final concentrations of 50 mM KCl and 10 mM MgCl_2 and incubated for 1 h at room temperature (~ 22 – 24°C) with or without 1.1 mM aspartate. Previous work with the serine receptor Tsr showed that the presence of 1 mM ligand did not change the extent of formation of kinase-activating signaling complexes relative to formation in the absence of ligand (Li and Weis, 2000). Thus the effects of the added aspartate can be attributed to effects on the activity of associated kinase. The phosphorylation reaction was initiated by addition of [γ -ATP] (63 $\mu\text{Ci}/\mu\text{mol}$) and terminated 15 s later by addition of 4X SDS sample buffer 980 mM Tris, 32 mM NaH_2PO_4 , pH 7.8, 4% (w/v) SDS, 4% (v/v) β -mercaptoethanol, 20 mM EDTA, 0.012 % (w/v) Bromphenol Blue, 40% (w/v) glycerol containing 20 mM EDTA. The amount of phospho-CheY produced was quantified by separation of the reaction mixture by SDS-polyacrylamide (15%) gel electrophoresis and phosphorimaging. Values for kinase activation were the difference between phospho-CheY produced in the presence of signaling complexes and the low amount produced in control reactions with Nanodiscs or native membrane lacking chemoreceptors ($\sim 1\%$ of maximal activity). For the experiments shown in Fig. 4A, final concentrations were 2 μM Tar, 0.1 μM CheA, 0.5 μM CheW and 1.6 μM CheY; for those shown in Fig. 5, they were, 0.25 μM CheA, 8 μM CheW, 20 μM CheY and the concentrations of Tar indicated.

Electron microscopy

Fractionated and clarified Nanodisc-inserted Tar preparations were diluted 1/10 in Tris-buffered saline (pH 7.4) and 5 μL samples placed on glow-discharged carbon/formvar electron microscopy grids. Grids were blotted with filter paper (Whatman #1) to remove excess sample and 5 μL of uranyl acetate (2%, w/v) applied and blotted after which the grid was air-dried. Grids were loaded into a Tecnai 12 electron microscope (FEI Corporation, Hillsboro, OR) equipped with a LaB6 filament operating at 120 kV. Projection images were recorded with a Gatan 2K charge-coupled device (Gatan, Inc., Abingdon, UK) at $\sim 25,000\times$ magnification.

Acknowledgments

We thank Wing-Cheung Lai for optimization of the kinase assay for Tar in native membrane and in Nanodiscs. This work was supported in part by grant GM29963 to GLH from the National Institute of General Medical Sciences and by funds to SS from the Center for Cancer Research, National Cancer Institute, NIH.

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Fig. 1. Chemoreceptor interactions and Nanodiscs

A. Cartoon of interactions among receptors. See text for details. Note that extended, tight clusters of receptors occur in conjunction with formation of signaling complexes with CheA and CheW, but for clarity those components are not shown in this representation of the cluster. B. A trimer of receptor homodimers formed from three dimers inserted in parallel into a Nanodisc. Important features of chemoreceptors are labeled. C. Representation of the result of independent inclusion of receptor dimers into Nanodiscs at an average content of ~3 dimers per disc.

Fig 2. Fractionation of a primary preparation of Tar-containing Nanodiscs

A representative fractionation on a size-exclusion column is shown for a primary preparation at 2.7 dimers per disc. A. Distribution of chemoreceptor Tar and membrane scaffold protein (MSP) plus the calculated value of Tar dimers per disc. Lines have been drawn to aid the eye. B. Protein content in each fraction as detected by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue

Fig 3. Nanodisc-embedded Tar in a native state as assessed by adaptational modification

In all panels, lines have been drawn to aid the eye. A. Modification of fractionated Tar-containing Nanodiscs. As a representative of multiple experiments with comparable results, measurements of maximal extent of methylation and deamidation are shown for the concentrated and clarified fractions of Fig. 2. B. Comparison of proportion of deamidation-active, Nanodisc-inserted Tar from primary preparations (Boldog *et al.*, 2006) and from fractionated, clarified material (data from Fig. 3A). C. Comparison of proportion of methylation-active, Nanodisc-inserted Tar from primary preparations (Boldog *et al.*, 2006) and from fractionated, clarified material (data from Fig. 3A).

Fig 4. Kinase activation by Nanodisc-embedded Tar

A. Kinase activation by fractionated, clarified Tar-containing Nanodiscs. As a representative of multiple experiments with comparable results, measurements of kinase activation, expressed as percent of activation by Tar in native membrane vesicles, are shown for the concentrated and clarified fractions of Fig. 2B as a function of the number of dimers per disc. Data points are means of three determinations with standard deviations as error bars. The line is a fit of the data to a modified Hill equation $A = A_0 + (A_S d^n) / (d_{50}^n + d^n)$ where A = the experimentally determined kinase activity, A_0 = the lower plateau level of kinase activity, A_S = the upper plateau level of enhanced kinase activity, d = the number of dimers per disc, d_{50} = the number of dimers per disc at which kinase activity was halfway between A_0 and A_S , and n = a parameter comparable to a Hill coefficient. For the fit shown, A_0 = 14%, A_S = 84% (and thus the upper plateau = 98%), d_{50} = 2.7 and n = 7.3. Representative data for kinase activity in the presence of a saturating concentration (1 mM) of the Tar ligand aspartate are shown for two other fractionations of primary Nanodisc-embedded Tar preparations. The line is drawn to aid the eye. Assays were at 2 μ M Tar, 0.1 μ M CheA, 0.5 μ M CheW and 1.6 μ M CheY in Disc Buffer containing 50 mM KCl and 10 mM MgCl₂. B. Comparison of kinase activation by Nanodisc-inserted Tar from primary preparations (Boldog *et al.*, 2006) (open circles; dashed line drawn to aid the eye) and fractionated, clarified material (closed circles, solid line; data and line from Fig. 3A).

Fig. 5. Kinase activation by Tar in Nanodiscs and in native membrane as a function of receptor amount

Signaling complexes were formed by incubating constant amounts of CheA and CheW with different amounts of Tar in a fractionated and clarified Nanodisc preparation at 4.7 dimers per disc or in native membrane vesicles. The plot shows the resulting mean kinase activity and standard deviation (two measurements for Tar in Nanodiscs and three for Tar in native membrane). For points with no visible error bars, the error was less than the diameter of the data point). Note that Tar:CheA was 20:1 for the experiments in Fig. 4A.

Fig. 6. Electron microscopy of Nanodisc-embedded Tar at ~1 Tar dimer per disc
Representative electron micrograph recorded from negatively stained preparations of fractionated, clarified Tar-containing Nanodiscs with an average of ~ 1.1 dimers per disc. Needle-like receptor dimers can be clearly seen in several Nanodiscs. Scale bar: 50 nm.