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His-Asp Phosphotransfer Signal Transduction[1]

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In general, protein phosphorylation is one of the most widely used mechanisms for regulating biological processes, including intracellular signal transduction. In eukaryotes, the cascades of protein phosphorylation and dephosphorylation events involving a number of protein tyrosine or serine/threonine kinases have been well studied. In contrast, recent intensive studies revealed that bacteria have devised a quite different phosphotransfer signaling mechanism for eliciting a variety of adaptive responses to their environment. Such a bacterial signal transduction mechanism was originally referred to as a "two-component regulatory system." The mode of molecular communication between a "sensor kinase" and its cognate phospho-accepting "response regulator" is principally based on histidine-to-aspartate (His-Asp) phosphotransfer. In *Escherichia coli*, for example, at least 30 different sensor-regulator pairs operate in a wide variety of adaptive responses. This particular signal transduction mechanism was once thought to be restricted to prokaryotes. However, many instances have recently been uncovered in diverse eukaryotic species. Furthermore, recent studies suggested that the molecular mechanism underlying the bacterial signal transduction is not simple as, and, in fact, is more sophisticated than thought previously. The new concept should be referred to as the "multi-step His-Asp phosphotransfer signaling mechanism."

Key words: adaptive response, phosphotransfer, regulator, signal transduction, sensor.

Bacteria have devised phosphotransfer signaling mechanisms for eliciting a variety of adaptive responses to their environment. They are collectively referred to as "two-component regulatory systems" (*1-6*, and references therein). A typical two-component system consists of two types of signal transducers, a "sensor kinase (or His-kinase)" and a "response regulator" (Fig. 1) (*2*). A sensor kinase monitors an environmental stimulus and modulates the function of a response regulator through protein phosphorylation. Accordingly, the response regulator mediates changes in gene expression or cell behavior. These signal transduction proteins contain one or both of the following common phosphotransfer signaling domains, "transmitter (or His-kinase)" and "receiver" domains (*5*). A typical sensor kinase has a transmitter domain, whereas a response regulator contains a receiver domain. A typical transmitter domain contains an invariant histidine residue, which is autophosphorylated in an ATP-dependent manner, while a typical receiver domain contains a conserved aspartate residue, which can acquire a phosphoryl group from the phospho-histidine of its cognate transmitter (*1*). Besides these two signaling domains, some signal transducer proteins were recently found to have another common domain, termed a "histidine-containing phosphotransfer (HPt) domain" (Fig. 1) (*7*, and references therein). In the most sophisticated case, a phosphoryl group moves from a transmitter to a receiver, and to a HPt domain, and finally to another receiver, during a signal transduction process. Thus, such a conceptual view as to the bacterial signal transduction mechanism should be principally referred to as the "multi-step His-Asp phosphotransfer signaling mechanism" (*7, 8*).

To date, instances of His-Asp phosphotransfer signaling systems have been reported in a large number of bacterial species (*4*). For example, inspection of the entire nucleotide sequence of *Escherichia coli* revealed that at least 30 different sensor-regulator pairs operate in this single species (*9*). This implies that the His-Asp phosphotransfer mechanism is a powerful device for a wide variety of adaptive responses in prokaryotic cells. Such a mechanism was once thought to be restricted to prokaryotes. However, it should be emphasized that many instances have recently been uncovered in diverse eukaryotic species, including yeasts, fungi, slime molds, and even higher plants (*10*, and references therein). This suggests that His-Asp phosphotransfer mechanisms may be involved in a wide variety of sophisticated signal transduction pathways in eukaryotes too.

Regarding such widespread His-Asp phosphotransfer signaling mechanisms, a number of thorough reviews have already appeared elsewhere (*1-10*). In this review, recent progress in this particular field will be discussed. First, I will describe the whole scenario with regard to the well-characterized EnvZ-OmpR signal transduction in response to the medium osmolarity in *E. coli*. Based on this classical example, a conceptual overview of the His-Asp phosphotransfer signaling mechanism will be given. Finally, intriguing instances of analogous multi-step His-Asp phos-

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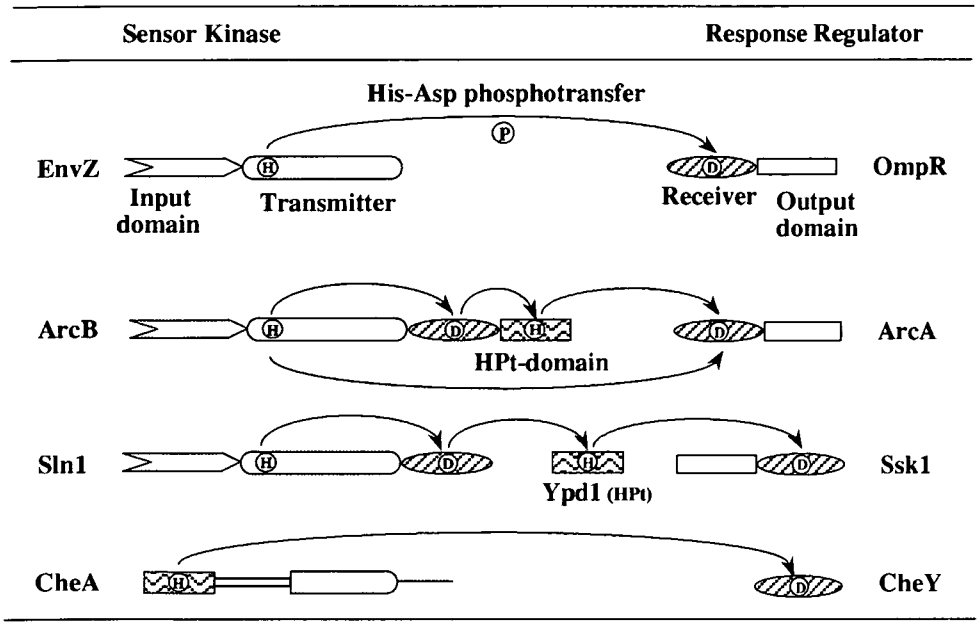

Fig. 1. **Schematic representation of the typical His-Asp phosphotransfer signaling between sensor kinase and response regulator.** The EnvZ/OmpR, ArcB/ArcA, and CheA/CheY systems operate in *E. coli*, and the Sln1/Ypd1/Ssk1 system operates in *S. cerevisiae*. Other details are given in the text.

photransfer signal transduction mechanisms in eukaryotic cells will also be discussed.

A simple example: EnvZ-OmpR phosphotransfer signaling in response to external osmolarity in *E. coli*

The ability of cells to adapt to external osmotic stress is a fundamental biological process that protects an organism against fluctuations in the water activity and solute contents of its environment. In *E. coli*, a set of genes whose expression is regulated by external osmotic changes is known (*11*). Among such types of osmotic responses, the expression of the major outer membrane proteins, OmpC and OmpF, has been the subject of extensive studies (Fig. 2) (*12*). Both the OmpC and OmpF proteins form passive diffusion pores in the outer membrane, which respectively facilitate the diffusion of small hydrophilic molecules across the membrane (*13*). Although they are very similar to each other in both structure and function, OmpF is preferentially synthesized in cells grown in a medium of low osmolarity, whereas OmpC is predominantly produced in a medium of high osmolarity, in such a manner that the total amounts in the outer membrane remain fairly constant. In genetic studies, the *ompC* and *ompF* loci have been identified as the structural genes for OmpC and OmpF, and mapped at 47 and 21 min on the *E. coli* genetic map, respectively. Two other genes, *envZ* and *ompR*, have also been found to be ones that regulate *ompC* and *ompF* expression at the transcription level (*14*). Together they play a crucial role in the complex mechanisms underlying the presumed osmo-responsive signal transduction and gene regulation. During the last two decades, intensive studies have been carried out to determine the molecular basis for this particular osmotic regulation, with special references to signal transduction and gene regulation in *E. coli*. As a result, the EnvZ-OmpR regulatory system is now one of the best understood instances of a His-Asp phosphotransfer signaling mechanism (Fig. 2).

EnvZ osmosensor kinase. EnvZ, consisting of 450 amino acids, is a cytoplasmic membrane protein containing two stretches of hydrophobic amino acids in its relatively N-terminal portion, which function as transmembrane anchors (*14–16*). Studies on the topology of EnvZ in the cytoplasmic membrane suggested that the N-terminal half of the EnvZ molecule, which is flanked by membrane-spanning segments, comprises an external periplasmic domain, whereas the C-terminal half comprises a cytoplasmic domain (*17*). In other words, its presumed catalytic domain protrudes into the cytoplasmic space, and the presumed signal-input domain projects into the external space. EnvZ functions as a dimer, in which a leucine zipper-like structure in the periplasmic domain plays an important role (*18*). While this receptor-like protein has been assumed to sense an environmental osmotic signal and then somehow to modulate the OmpR function, little was known for a long time about the biochemical event that takes place during the presumed transmembrane signaling. Two important findings then shed light on this problem. First, a number of bacterial proteins were found to exhibit significant similarity to the amino acid sequence of the C-terminal half of EnvZ (*ca.* 240 amino acids), and these proteins were also assumed to function as sensors for environmental stimuli (*e.g.*, CheA chemotaxis-sensor, NtrB nitrogen-sensor, and PhoR phosphate-sensor) (see Table I) (*1*). More importantly, its was demonstrated *in vitro* that a truncated form of EnvZ comprising only the C-terminal half has the ability to undergo autophosphorylation at a certain histidine residue (His-243) in the presence of ATP (*19–21*). Similar activity was demonstrated simultaneously for other members of the EnvZ-like protein family mentioned above (*3*). These common domains, which are analogous in both structure and function, are now generally referred to as "transmitters." These domains exhibits histidine-kinase activity in common. In short, EnvZ turned out to be an osmo-responsive kinase.

OmpR response regulator. OmpR, consisting of 239 amino acids, is a cytoplasmic protein and comprises two functional domains: the N-terminal domain is responsible for the interaction with EnvZ, and the C-terminal one for

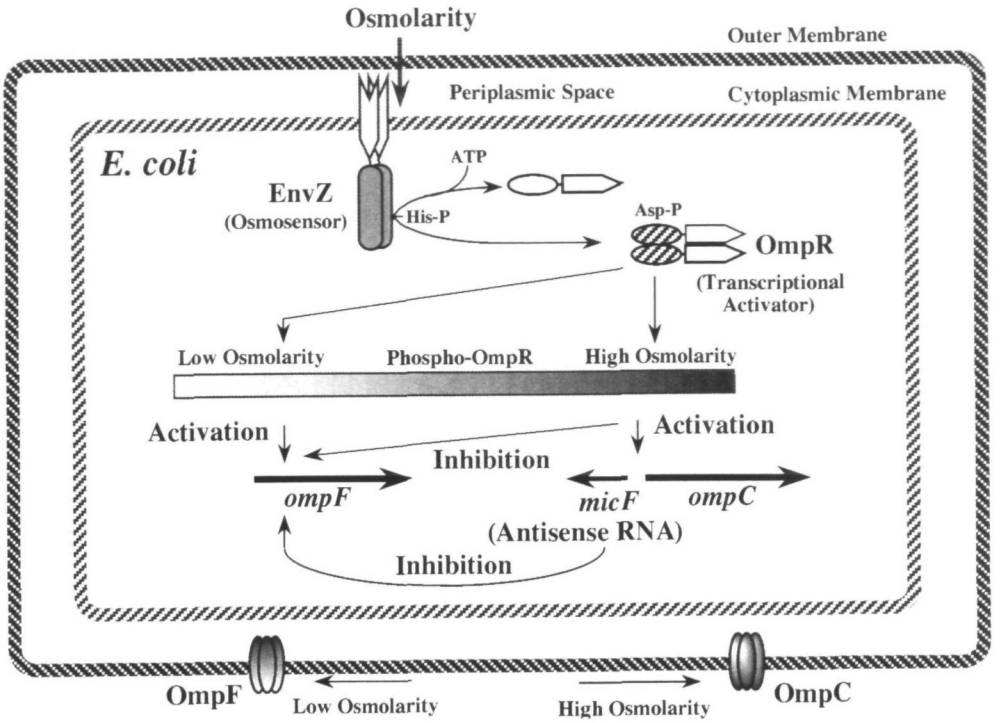

Fig. 2. Schematic representation of the molecular mechanism underlying the osmotic regulation of the expression of the outer membrane proteins, OmpC and OmpF, through the EnvZ-OmpR phosphotransfer signal transduction in *E. coli*. Details are given in the text.

the interaction with DNAs in the *ompC* and *ompF* promoters (Fig. 1) (*22, 23*). In this sense, OmpR is a transcritional activator, which can help RNA polymerase to trigger the transcription. The *cis*-acting upstream sequences, *i.e.*, the OmpR-binding sites, in both the *ompC* and *ompF* promoters cover stretches of more than 60 nucleotides, which provide at least three distinct OmpR-binding sites in each case (*24, 25*). These multiple OmpR-binding sites probably together play a role in the complex mechanism underlying the osmotic regulation of *ompC* and *ompF* (see below).

As in the case of the EnvZ-like proteins, the amino acid sequence of the N-terminal domain of OmpR exhibits extensive similarity with those of a number of known bacterial proteins, including CheY chemotaxis-regulator, NtrC nitrogen-regulator, and PhoB phosphate-regulator (see Table I) (*1*). *In vitro*, the N-terminal half of OmpR is capable of undergoing phosphorylation in the presence of EnvZ and ATP (*23*). The phospho-accepting amino acid was found to be a certain aspartate residue (Asp-55), which is located around the center of the N-terminal half. The phosphorylated form of OmpR is then capable of undergoing dephosphorylation, which is stimulated by EnvZ and ATP (*26*). In this sense, EnvZ exhibits two activities toward OmpR: apparent kinase and phosphatase activities specific for OmpR. Similar events have also been observed for other analogous members of the regulator family mentioned above (CheY, NtrC, and PhoB) (*3*). These proteins are also phosphorylated similarly by their cognate sensor kinases (*i.e.*, CheA, NtrB, and PhoR), respectively. These common phospho-accepting domains are generally referred to as "receivers" (*4*). In short, OmpR turned out to be a transcriptional activator, whose function is modulated through phosphorylation.

It can be easily accepted that once the N-terminal receiver domain of OmpR is phosphorylated by EnvZ, the DNA-binding properties of the C-terminal half may be altered through a conformational change (*27*). In fact, when the purified OmpR protein is phosphorylated *in vitro*, it undergoes dimerization, which results in great enhancement of its ability to bind to specific target DNAs (*28*). Consequently, the phosphorylated OmpR-dimer functions as a transcriptional activator for both the *ompC* and *ompF* promoters. It is worth mentioning that *E. coli* has at least 14 members of the OmpR family of transcriptional activators (*e.g.*, ArcA, KdpE, and PhoB), whose amino acid sequences show extensive similarity to each other not only in the N-terminal receiver domains but also in the C-terminal DNA-binding domains, yet each protein regulates a specific set of target genes (Table I) (*29*). The occurrence of members of the OmpR family has also been reported in many other bacterial species (even in chloroplasts), suggesting that the OmpR-like proteins are some of the most widespread DNA-binding transcriptional regulators in prokaryotes (*4*).

The structure of the common DNA-binding domain of OmpR, determined by X-ray crystallography, is now available (*30, 31*). The X-ray structure revealed that the DNA-binding domain of OmpR has 3 α helices, preceded by a β sheet, 2 of which (α2 and α3) create together a structure very similar to the canonical helix-turn-helix (HTH) DNA-binding motif. However, this presumed HTH-structure of OmpR is unique in that it has a large loop consisting of 11 amino acids between α2 and α3. This may be the reason why previous inspection failed to detect a putative HTH-motif in OmpR. Another interesting fact is that the DNA-binding domain of OmpR contains a direct contact site(s) for RNA polymerase, presumably, for the α-subunit (*32*). In this respect, the results of genetic studies suggested the large loop in the HTH-motif may be

TABLE I. **Compiled list of ORFs involved in phosphotransfer signaling in *E. coli*.**

Sensor/regulator[a]	gb: Accession[b]	Relevant adaptive systems, [putative][c]
OmpR-family		
PhoR/PhoB	AE000146	Phosphate regulation
f480/f227	AE000162	[Cops/R, *Pseudomonas*, copper response]
KdpD/KdpE	AE000173	Potassium transport
TorS/TorR	AE000201	Trimethylamin metabolism
PhoQ/PhoP	AE000213	Stress situations
RstB/RstA	AE000256	?
f452/f239	AE000288	[CzcS/C, *Alcaligenes*, heavy metal homeostasis]
BaeS/BaeR	AE000297	[AFQ2/1, *Streptomyces*, second metabolism]
o449/o219	AE000384	[YgiH/X, *Haemophilis*, unknown]
ArcB/ArcA	AE000400/510	Respiratory control
EnvZ/OmpR	AE000416	Osmotic regulation
CpxA/CpxR	AE000466	Maltiple systems
BasS/BasR	AE000483	Virulence
CreC/CreB	AE000510	Catabolite repression
NarL-family		
−/Fimz	AE000159	[−/FimZ, *Salmonella*, fimbrial expression]
NarX/NarL	AE000220	Nitrate regulation
−/UvrY	AE000284	?
Rcsc/RcsB	AE000311/310	Capsule synthesis
Evgs/EvgA	AE000325	[Bvgs/A, *Bordetella*, virulence]
NarQ/NarP	AE000333/309	Nitrate regulation
UhpB/UhpA	AE000444	Hexose phosphate uptake
Ntrc-family		
AtoS/AtoC	AE000311	Acetoacetate metabolism
f496/f444	AE000342/341	?
NtrB/NtrC	AE000462	Nitrogen regulation
HydH/HydG	AE000473	Labile hydrogenase activity
CheY-family		
CheA/CheB/CheY	AE000282	Chemotaxis
Others-A		
o552/o226	AE000167	[−/CriR, *Shigella*, *ipa* genes expression]
YjdH/YjdG	AE000485	[CitA/B, *Klebsiella*, citrate metabolism]
Others-B		
YehU/YehT	AE000301	[Lyts/T, *Bacillus*, autolysin response]
o565/o244	AE000326	[−/MrkE, *Klebsiella*, fimbrial expression]
Others		
−/RssB	AE000222	Sigma-S degradation
YojN	AE000310	?
BarA/−	AE000362	Pilus adherence

[a]Cognate pairs of sensor/regulator are classified and listed. [b]The GenBank accession numbers for the seqeunces of the set of 400 *E. coli* chromosomal segments are indicated, in each of which the corresponding gene(s) is located. [c]The anotations in the parentheses are solely putative, and are based on similarities to the members of other spices, as indicated.

involved in this interaction with RNA polymerase, thereby serving as a transcriptional activation domain (*30, 31*).

A scenario for the molecular mechanism underlying the EnvZ-OmpR signal transduction. Based on the various lines of experimental evidence described above, together with those described elsewhere, one can envisage the following molecular mechanism underlying the differential activation of the *ompC* and *ompF* genes in response to the medium osmolarity (Fig. 2) (*12*). First, the EnvZ-dimer, with a receptor-like structure, in the cytoplasmic membrane senses an environmental osmotic stimulus. In this process, the N-terminal membrane-spanning and periplasmic domains of the EnvZ-dimer presumably can take on two alternative conformational states (*i.e.*, a high osmolarity form and a low osmolarity form, respectively). This presumed conformational change may be, for example, due to a change in the relative orientation of each single EnvZ molecule in the dimer. The primary signal for such a conformational change may be that caused by a change in the physical membrane-tension that is created in the cytoplasmic membrane through the osmotic pressure. This idea is supported by the fact that certain amphipaths (*e.g.*, procaine and chlorpromazine) are capable of mimicking the osmotic signal, when added to the low osmolarity medium at a low concentration (*15, 16*). In any event, in the high osmolarity state, EnvZ actively undergoes autophosphorylation at histidine residue-243 in the C-terminal kinase domain, and then efficiently transfers its phosphoryl group to the N-terminal receiver domain of OmpR at aspartate residue-55. Upon phosphorylation, OmpR becomes an active dimer that exhibits enhanced DNA-binding ability specific for both the *ompC* and *ompF* genes. In the low osmolarity state, however, EnvZ exhibits relatively low kinase activity (*i.e.*, high phosphatase activity) toward OmpR. Such osmotic modulation of the kinase/phosphatase activity of EnvZ results in that the relative amounts of the phosphorylated form of OmpR in cells varies in response to the medium osmolarity. When the medium osmolarity is low, the relative amount of the phosphorylated form of OmpR in cells is relatively small. In this particular situation, the *ompF* gene is first triggered, because the *ompF* promoter has relatively high-affinity OmpR-binding sites.

As the medium osmolarity increases, the relative amount of the phosphorylated form of OmpR increases proportionally, which in turn results in preferential activation of the *ompC* gene with low-affinity OmpR-binding sites. The transcription of the *ompF* gene is inhibited when the amount of the phosphorylated form of OmpR is rather high, because this promoter also has low-affinity OmpR-binding sites, at which OmpR functions as a transcriptional repressor (*33*). An antisense RNA (MicF) is also responsible for this repression, presumably, at the posttranscriptional level (*34*). As a whole, one can now observe a curious phenomenon: when *E. coli* is grown in a low osmolarity medium, its outer membrane is enriched with OmpF, whereas OmpC is predominant in a high osmolarity medium. This EnvZ-OmpR regulatory system is a simple and typical example of bacterial signal transduction through His-Asp phosphotransfer protein phosphorylation.

Not so simple example: sophisticated His-Asp phosphotransfer signaling mechanism

As mentioned above as a simple example, the EnvZ osmosensor has a C-terminal histidine-kinase transmitter domain, which is preceded by a unique N-terminal region that presumably functions as an osmo-sensing domain (signal-input domain) (Fig. 1). Its cognate OmpR response regulator contains an N-terminal phospho-accepting receiver domain, which is followed by a specific region that serves as a DNA-binding domain (signal-output domain). Similarly, many His-Asp phosphotransfer signal transducers contain one of the common domains, a transmitter or receiver (*4*). As a very simple view, a sensor containing a transmitter can signal a regulator containing a receiver through His-Asp phosphotransfer. The majority of sensor-regulator pairs, so far uncovered in a large number of bacterial species, have this type of simple signaling design. This is why such bacterial signal transduction systems are collectively referred to as "two-component systems." However, the reality is not so simple as thought previously, because another common histidine-containing phosphotransfer (HPt) signaling domain was recently discovered in some signal transducers (Figs. 1 and 3) (*7, 35, 36*).

As shown in Fig. 1, the ArcB sensor functions together with the ArcA regulator in an aerobic/anaerobic regulatory circuit in *E. coli*. The presumed ArcB redox-sensor is an extreme instance in that it contains all of the three types of common signaling domains in its primary sequence (*35*). It was demonstrated that a multi-step His-Asp phosphotransfer reaction occurs within this single protein, from His-292 to Asp-576, and subsequently to His-717 (either in an intra- or inter-molecular manner). The phosphoryl groups in ArcB are finally transferred from either His-292 or His-717 to the receiver domain (Asp-54) of the cognate ArcA regulator. The ArcB sensor is not unique in the sense that there are many examples of signal transducers that contain two or more of the signaling domains within a single polypeptide (*7*). They are collectively designated as "hybrid sensor kinases." *E. coli* alone has at least 5 hybrid kinases (BarA, EvgS, RcsC, and TorS), which have a structural design very similar to that of ArcB (*9*). More (16 hybrid sensor kinases) were found in a photosynthetic cyanobacterium (*Synechocystis* sp.) (*37*). An even more striking example recently reported was the osmo-responsive signal transduction of the eukaryotic microorganism, *Saccharomyces cerevisiae* (Fig. 1) (*38*). The three components, Sln1p (sensor kinase)-Ypd1p (HPt domain)-Ssk1p (response regulator), are involved together in the His-Asp phosphotransfer signaling pathway. This pathway is initiated by the autophosphorylation of the Sln1p osmosensor in the transmitter domain. This phosphate is transferred to its own receiver domain, then transferred to the Ypd1p-HPt domain, and finally to the receiver domain in Ssk1p. Interestingly, Ypd1p is a separate polypeptide and only contains a HPt domain. It appears to serve solely as a mediator of the His-Asp phosphotransfer. In terms of His-Asp phosphotransfer signaling, the mechanistic significance of such a complicated signaling design is not fully understood (*7*). This issue will be addressed later.

In any case (two-components or more), a phosphoryl group moves sequentially from a histidine/aspartate residue in a domain to an aspartate/histidine residue in another domain, resulting in the modification of the final target through phosphorylation. The final target is generally a response regulator containing a receiver domain, which functions as a biological molecular switch, whose ability is modulated by its phosphorylation state (the phosphorylated form may be an active form, or, the non-phosphorylated form may be so). Consequently, many biological processes can be controlled in a specific manner through this common phosphotransfer mechanism.

More than two components: multiple His-Asp phosphotransfer signaling domains

Transmitter (His-kinase). A typical transmitter domain of about 240 amino acids contains several short stretches of amino acids, which are almost invariantly conserved among a family of proteins (Fig. 3) (*1, 4*). Such a transmitter exhibits histidine kinase activity to autophosphorylate its own histidine residue (H1), which is located in a relatively N-terminal region within the transmitter domain. It is worth mentioning that this phosphorylation event may take place in an intermolecular fashion. In any case, this histidine residue is an exclusively conserved signature, with the one exception of the CheA family of

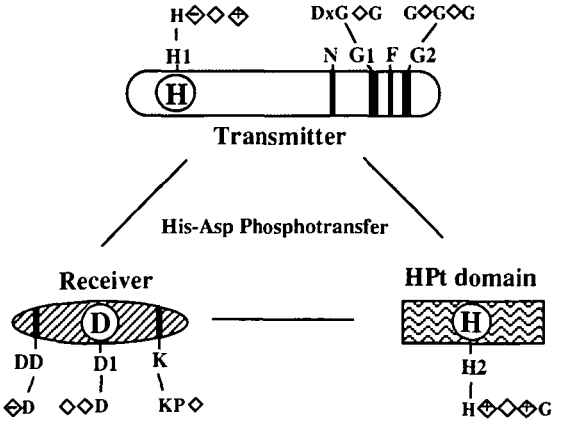

Fig. 3. **Structural feature of the transmitter, receiver, and HPt domains.** Conserved signature motifs are schematically shown for the three kinds of phosphotransfer signaling domains. Letters indicate amino acids present in at least 70% of aligned domains. Diamonds indicated positions at which at least 50% of the amino acids belong to the same chemical family: white, nonpolar; plus sign, basic; minus sign, acidic or amidic. Other details are given in the text.

sensor kinases (Fig. 1). In any case, other conserved amino acids are characterized by an asparagine residue (N), a phenylalanine residue (F), and 2 glycine-rich motifs (G1, DxGxG; G2, GxGxG), which should be located at appropriate distances relative to each other. The active histidine residue is autophosphorylated at position N-3 of the imidazole ring through the formation of a phosphoamidate in an ATP-dependent manner. The two glycine-rich motifs may be involved in the ATP-binding. Once autophosphorylated, the transmitter domain serves as a relatively high energy phospho-donor for its cognate receiver. The specificity of this His-Asp phosphotransfer in each sensor-regulator pair appears to be reasonably high, so that cross-talk between non-cognate pairs of transmitters and receivers is usually not observed *in vivo*. The basis of this specificity remains unclear.

Receiver. A typical receiver domain of about 120 amino acids contains several short stretches of conserved amino acids (Fig. 3) (*1, 4*). Such a receiver exhibits phospho-accepting ability to be modified at its aspartate residue, which is located around the center. Together with this critical aspartate residue (D1), 2 other signature amino acids are exclusively conserved among the receiver family; another aspartate residue (DD) at the N-terminal end, and a lysine residue (K) at the C-terminal end. The three-dimensional structures have been determined in a few instances (*e.g.*, CheY, NarL, and Spo0F). The first characterized CheY chemotaxis-response regulator and also others have an α/β barrel comprising five sets of alternating β strands and α helices connected by short loops or turns (*4*, and references therein). The conserved amino acids (Asp-12, Asp-13, Asp-57, and Lys-109 in CheY) lie close together, forming a pocket in which Asp-57 is presumably phosphorylated (note that the determined structures are for the unphosphorylated state). The aspartate residue is modified through the formation of an acyl phosphate on acquisition of the phosphate from the phospho-histidine of its cognate transmitter. This phosphotransfer reaction appears to be catalyzed by the receiver itself. In fact, instances have been reported of some receivers that can phosphorylate themselves by using small molecular phospho-donors including acetyl phosphate and phosphoramidate, although the *in vivo* significance of this is unclear (*4*). The phosphorylated receiver is relatively unstable, and is readily converted to its unphosphorylated form (the *in vitro* half-lives of phospho-CheY and phospho-OmpR are a few seconds and several hours, respectively) (*3*). This dephosphorylation process also appears to be autocatalytic. In some cases, however, the transmitter domain may modulate this process to control the phosphorylation state of the cognate receiver, as described in the case of EnvZ-OmpR (*26*). In any event, such rapid and controlled conversion of the phosphorylation/dephosphorylation state of a certain receiver is obviously crucial for the underlying signal transduction mechanism, because the state of a receiver may directly control its own biological activity by covering the ON/OFF state of the covalently attached signal output domain, as mentioned above (*4*).

HPt domain. The histidine-containing phosphotransfer (HPt) domain was discovered very recently, but now is known to play an important role in some (but not all) His-Asp phosphotransfer signaling systems (*7*). In a given sequence of a signal transducer, the HPt domain is the most difficult to recognize, because its amino acid sequence is quite variable, and does not resemble that of the authentic histidine site of the transmitter (the BLAST and FASTA search programs may not always be helpful to find a HPt domain) (*35*). In a typical HPt domain, however, an invariant and phosphorylated histidine residue (H2) is followed by a short characteristic stretch of amino acids (Fig. 3). The X-ray crystal structure of the HPt domain of ArcB revealed that it consists of 6 α helices including a 4-helix bundle with a kidney-like shape (*39*). This determined structure supported the reliability of the short consensus sequence proposed previously for the HPt-family of domains. Based on this sequence criterion, a number of bacterial signal transducers were predicted to contain such a domain (*e.g.*, ArcB and BarA of *E. coli*, BvgS of *Bordetella pertussis*, and Ypd1p of *Saccacharomyces cerevisiae*) (*7, 35*). Unlike authentic transmitters, the HPt domain does not exhibit any catalytic function. The HPt domain thus appears to serve solely as a passive intermediate molecule (or substrate) in His-Asp phosphotransfer by acquiring/transferring a phosphoryl group from/to another signaling domain. The phosphoryl group incorporated into the HPt domain is very stable (at least *in vitro*), and thus the cognate receiver is enzyme in its own right, capable of undergoing autophosphorylation.

Why so many pathways: *E. coli* has more than 30 His-Asp signaling pathways

The recently determined entire genomic sequence of *E. coli* allowed us to compile a complete list of genes presumably encoding His-Asp phosphotransfer signal transduction proteins (*9*). This revealed the occurrence of 23 orthodox sensor kinases, 5 hybrid sensory kinases, and 32 response regulators, in this single bacterial species. This indicates that about 30 distinct His-Asp phosphotransfer signaling pathways operate in response to a wide variety of environmental stimuli in *E. coli* (Table I).

Twenty-three ORFs were identified as orthodox sensor kinases containing a transmitter in which a typical transmitter domain is preceded by a presumed N-terminal signal-input domain. However, the signal-input domain of each of these sensor kinases has its own context with regard to amino acid sequence and length, therefore each may serve as a specific signal transducer. This bacterium has 5 hybrid sensory kinases, RcsC, TorS, ArcB, EvgS, and BarA. RcsC contains both transmitter and receiver domains, while the others contain a HPt domain too. All of the 32 response regulators of *E. coli*, except for CheY, have a similar structural design, *i.e.*, a common receiver domain is followed by a specific output domain. CheY consists of only a receiver domain (Fig. 1). Cross-examination of the sequences of the respective signal-output domains revealed that they can be classified into distinct subgroups. Fourteen ORFs exhibit extensive similarity to OmpR not only in the receiver domains, but also in the output domain (OmpR subfamily). Seven others appear to be members of the NarL subfamily and 4 other ORFs are members of the NtrC subfamily. It should be noted that the OmpR, NarL, and NtrC response regulators are, respectively, known to function as specific DNA-binding transcriptional regulators. It can thus be assumed that all these 25 members are DNA-binding transcriptional regulators. These response regulators are implicated in gene regulation in response to

some environmental parameters, yet each of them is postulated to regulate (activate or repress) a specific subset of genes. The CheB and RssB regulators are unique in that each has a unique signal-output domain. The former exhibits esterase activity involved in chemotactic signal transduction, whereas the latter is somehow involved in the proteolytic degradation of the sigma-factor (σ^S) through the function of ClpXP protease.

The chromosomal positions of the coding sequences specifying these signal transducers are scattered evenly over the *E. coli* genome. In most instances, however, a certain cognate sensor/regulator pair are located next to each other. Of these putative pairs of sensors/regulators, in 17 cases, the physiological functions in certain adaptive responses were documented previously to some extent (Table I). They include, for example, signal transduction systems involved in substrate-transport and assimilation of phosphate (PhoR/PhoB), potassium (KdpD/KdpE), nitrogen (NtrB/NtrC), and hexose phosphate (UhpB/UhpA); ones involved in respiratory control (ArcB/ArcA, NarX/NarL, and TorS/TorR); ones involved in virulence (BasS/BasR, RcsC/RcsB, and BarA), ones involved in chemotactic motility (CheA/CheY/CheB); *etc.* The remaining sensors/regulator pairs and two solo regulators remain to be characterized, although some of them exhibit sequence similarity to known sensors/regulators from other bacterial species (Table I).

To date, the entire genomic sequences have been completed for 10 prokaryotic microorganisms, including *Archaea* (*40*, and references therein). A brief inspection of these databases revealed that *Haemophilus influenzae* contains 4 sensors-5 regulators; *Haelicobacter pylori*, 4 sensors-7 regulators; *Synechocystis* sp., 32 sensors-38 regulators; *Mycoplasma gentitalium*, none; *Methanococcus jannashii* (archaebacterium), none; and *Methanobacterium thermoautotrophicum* (archaebacterium), 15 sensors-9 regulators. *Bacillus subtilis* also appears to contain a number of sensor-regulator pairs (as many as *E. coli*). Prokaryotic microorganisms that are supposed to be exposed to more variable environments may have more His-Asp phosphotransfer signaling systems. Thus, the His-Asp phosphotransfer signal transduction is very common in prokaryotes.

Not just in prokaryotes: His-Asp phosphotransfer signaling systems in eukaryotes

Many instances of His-Asp phosphotransfer signaling systems were recently discovered in diverse eukaryotic species including yeasts, fungi, slime molds, and higher plants (*10*, and references therein). The best characterized example is the budding yeast osmo-responsive system, as briefly mentioned above (Fig. 4A, and see Fig. 1) (*38, 41*). In *Neurospora crassa*, a gene encoding a hybrid sensor kinase (NIK1) has been reported (*42*). In *Dictyostelium discoidium*, a few hybrid sensor kinases have also been identified (DokA, DhkA), one of which may also be involved in the osmoregulation in this organism (*43, 44*). In the higher plant, *Arabidopsis thaliana*, 3 sensor kinases each containing a typical transmitter domain have so far been discovered, namely, ETR1, ERS, and CKI1 (an ETR1- like sensor, NR, was also found in tomato) (*45-48*). The ETR1 and CKI1 sensors contain a receiver domain at their relatively C-terminal ends. Thus, they resemble the yeast Sln1p osmosensor. Both the ETR1 and ERS kinases function as ethylene (plant hormone) receptors, while the CKI1 kinase is presumably involved in the cytokinin (plant hormone) signal transduction pathway (Fig. 4B). Based on these facts, one can reasonably suppose that His-Asp phosphotransfer signaling systems may operate in *Arabidopsis*. If so, it should also have other signal transducers that function as either response regulators or HPt domains. In this respect, we have recently demonstrated that *Arabidopsis* possesses a group of response regulators that contain a functional phospho-accepting receiver domain. We cloned 5 distinct *Arabidopsis* genes each encoding a response regulator (named ARR), suggesting that multiple His-Asp phosphotransfer pathways exist in this higher plant (Fig. 4B). These instances collectively imply that the His-Asp phosphotransfer mechanism is a universal mode of signal transduction in both prokaryotes and eukaryotes.

Link between the MAPK cascade and His-Asp phosphotransfer. In yeast osmotic-adaptation, the intracellular accumulation of compatible osmolytes such as glycerol appears to be important. In the budding yeast, *S. cerevisiae*, a key gene is GPD1 encoding NAD^+-dependent glycerol-3-phosphate dehydrogenase, whose expression is remarkably induced when cells are exposed to high osmolarity (Fig. 4A). Originally, a MAP (mitogen-activated protein) kinase cascade was demonstrated to be involved in this osmosensing signal transduction (*10, 38, 41*, and references therein). This particular MAPK cascade is

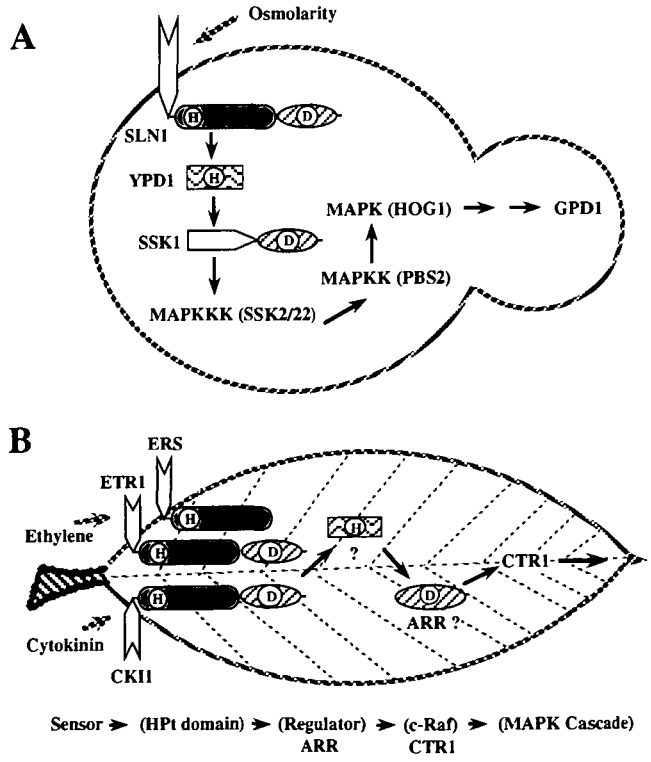

Fig. 4. **Schematic representation of the His-Asp phosphotransfer signaling pathways found in eukaryotes.** (A) An osmotic adaptation of *S. cerevisiae* was proposed to be under the control of the Sln1-Ypd1-Ssp1 phosphotransfer signaling pathway, which links to the HOG1-MAPK cascade. (B) Plant hormone responses in *Arabidopsis* also appear to be mediated by the His-Asp phosphotransfer signaling mechanism. Other details in the text.

termed the HOG1 (high osmolarity glycerol response) MAPK cascade. This signaling system consists of Ssk2p/Ssk22p (a pair of redundant MAPKKKs), Rbs2p (MAPKK), and HOG1p (MAPK). A striking finding was that the above mentioned His-Asp phosphotransfer signaling pathway, Sln1p-Ypd1p-Ssk1, is located upstream of this typical enkaryotic MAPK cascade. The Ssk1 response regulator interacts directly with Ssk2p/Ssk22p, and presumably modulates the activity of these MAPKKKs, depending on the phosphorylation state of the Ssk1 receiver. A similar osmosensing pathway seems to operate also in the fission yeast, *Schizosaccharomyces pombe*. Putative histidine kinases (MAK1/MAK2) and a response regulator (Mcs4) were reported recently for *S. pombe* (*10*).

Another instance of a link between His-Asp phosphotransfer signaling and the MAPK cascade was suggested in the ETR1 ethylene-responsive pathway of *Arabidopsis* (Fig. 4B) (*45, 46*, and references therein). The CTR1 gene encoding a protein kinase homologous to mammalian *c*-Raf kinase is believed to act downstream of the ETR1 histidine kinase. Because the mammalian *c*-Raf kinase is an activator of a MAPKK, it is tempting to speculate that the MAPK cascade is involved in the ethylene-responsive signaling pathway, which is initiated by the ETR1 His-kinase. These facts suggest that the prokaryotic type of phosphotransfer signal transduction mechanism and the eukaryotic type of protein phosphorylation cascade are fully compatible in a cell to perform in concert a sophisticated task in intracellular signal transduction.

Why it takes multi-steps: Future problems remaining

The discovery of multi-step His-Asp phosphotransfer raised the new question of what is the advantage of multi-step phosphotransfer signaling, since this particular mechanism does not serve to amplify signals (in contrast to the MAPK cascade). This issue has previously been addressed, yet no clear view has emerged (*7, 10*). It may provide the potential for the integration of multiple signals at intermediate steps (*e.g.*, cross-talk between multiple pathways), as has been discussed in the case of the Kin-Spo0 pathway in *Bacillus subtilis* (*6*). Alternatively, the extra His-Asp phosphotransfer components may serve as multiple regulatory checkpoints in the signaling pathway. As an example, certain phosphatases have been implicated in some two-component systems (*49*). RapA and RapB of *B. subtilis* are known to be phosphatases specific to the Spo0F response regulator (*i.e.*, phospho-aspartate phosphatase). The dephosphorylation of phospho-aspartate on the CheY chemotactic response regulator is also modulated by CheZ phosphatase (*3*). Similarly, one can envisage that the phosphohistidine in a HPt domain would also be a potential and alternate target of regulatory phosphatases. Such a phospho-histidine phosphatase, if present, should also serve as a modulator for the His-Asp phosphotransfer. We recently identified a candidate (named SixA) that interacts with the HPt domain of the ArcB sensor kinase, through its dephosphorylation ability (*50*). The combined results of *in vivo* and *in vitro* studies as well as a computer-aided inspection strongly supported the view that the SixA protein has phospho-histidine phosphatase activity, which may be involved in the modulation of the His-Asp phosphotransfer. As mentioned above, 30 His-Asp signaling pathways operate in *E. coli*. Are they working specifically and independently? Are they forming a network of signaling pathways, by adopting unknown mechanisms such as "cross-talk," "multi-signals integration," "signal feedback," "adaptation," and "modulation through phosphatases"? Clarification of these crucial issues with regard to the His-Asp phosphotransfer signaling must await further intensive studies.

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