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COMMENTARY

Eukaryotic signal transduction via histidine-aspartate phosphorelay

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SUMMARY

Transmembrane signal transduction is a feature common to all eukaryotic and prokaryotic cells. We now understand that a subset of the signalling mechanisms used by eukaryotes and prokaryotes are not just similar in principle, but actually use homologous proteins. These are the histidine-aspartate phosphorelays, signalling systems of eubacterial origin, now known to be widespread in eukaryotes outside the animal kingdom. Genome projects are revealing that His-Asp phosphorelays are present as multigene families in lower eukaryotes and in plants. A major challenge is to understand how these 'novel' signal transduction systems form integrated networks with the more familiar signalling mechanisms also present in eukaryotic cells. Already, phosphorelays have been characterised that regulate MAP kinase cascades and the cAMP/PKA pathway. The probable absence of His-Asp phosphorelays from animals has generated interest in their potential as targets for anti-microbial therapy, including antifungals. Recent findings suggest that this approach holds promise.

Key words: His-Asp phosphorelay, Signal transduction, Histidine kinase, Response regulator, RegA, MAP kinase, *Dictyostelium*

AN UNEXPECTED DISCOVERY

A little more than five years ago the discovery of His-Asp phosphorelay signal transduction in eukaryotes took everyone by surprise. Until then it was thought that this ancient signalling mechanism was restricted to prokaryotes (Parkinson, 1993). This was a reasonable presumption: hundreds of the things had been found in bacteria, but, despite our cleverness in dissecting signal transduction pathways in eukaryotes, we had not come across them there. Histidine phosphorylation was known to occur in eukaryotic cells (a phosphorylated histidine residue is an intermediate in several enzymatic reactions), but this was clearly different from the signalling mechanisms known in bacteria (Pirrung, 1999). This changed when two groups discovered phosphorelays critical for environmental responses in yeast and plants (Chang et al., 1993; Maeda et al., 1994). Since that time many more phosphorelay genes have been discovered in diverse eukaryotes. It is important to point out, however, that no such genes have been identified in animals. The published genomes of *Caenorhabditis elegans* (*C. elegans* sequencing consortium, 1998) and *Drosophila melanogaster* (Adams et al., 2000) do not contain any phosphorelay genes, and the human genome project has not as yet revealed any candidates.

WHAT IS HIS-ASP PHOSPHORELAY?

Histidine-aspartate phosphorelays consist of a series of signal transduction proteins that alternately possess a site of histidine or aspartate phosphorylation, or both. The chemistry of the phosphoramide and acylphosphate linkages involved is distinct from that of the O-phosphate linkages of phosphoserine, phosphothreonine and phosphotyrosine (Fig. 1).

His-Asp phosphorelay pathways contain up to three types of signalling element. Firstly, a histidine protein kinase (HK), which contains a site of histidine phosphorylation, acts as a transmitter. The HK functions as a dimer, transphosphorylating its partner subunit, using the γ-phosphoryl group of ATP (Surette et al., 1996; Bilwes et al., 1999). The HK communicates with a response regulator protein that possesses a receiver domain containing a conserved site of aspartate phosphorylation. The response regulator protein harbours the cellular output activity of the phosphorelay pathway. Aspartate phosphorylation of the response regulator acts as a molecular switch to control output activity, thus transducing the original signal (Parkinson, 1993). A third module, the histidine phosphotransfer (HPt) domain, allows different receiver domains to communicate with each other in more complex phosphorelays (Fig. 2).

Histidine kinase activity is regulated by receptor-ligand interactions. In many cases, histidine kinases themselves are transmembrane receptors that have an N-terminal sensing domain; in other cases, the histidine kinase lies downstream of a separate receptor. Because many histidine kinases also act as phosphatases for their cognate response regulators (Perego and Hoch, 1996), ligand binding can alter the balance of kinase and phosphatase activities and therefore act as an efficient

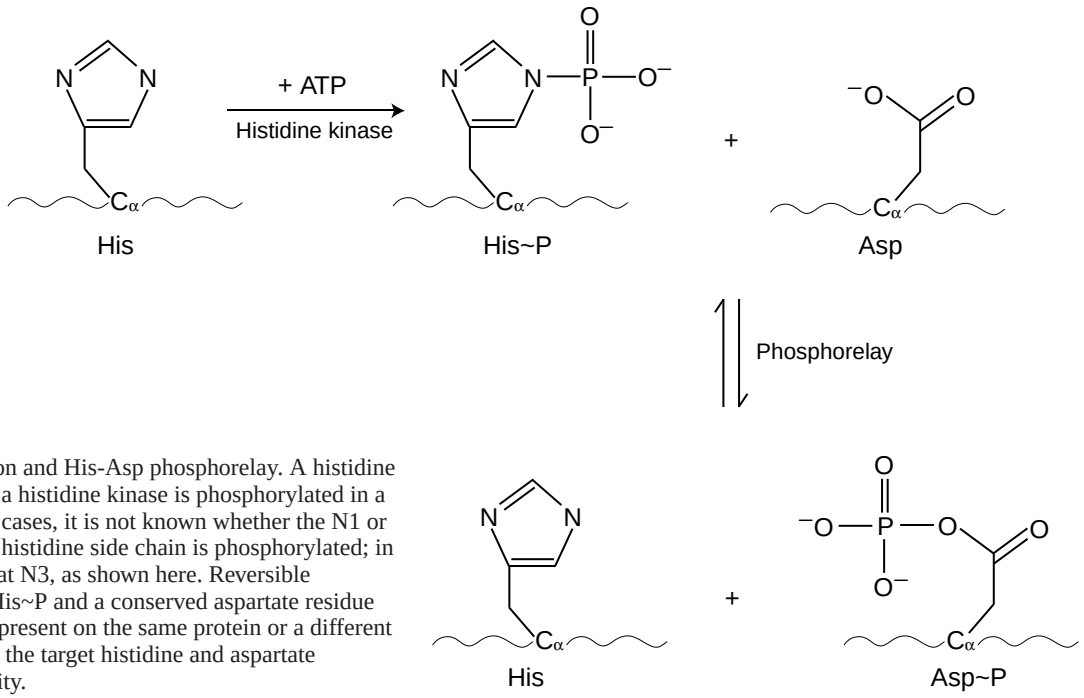

Fig. 1. Histidine phosphorylation and His-Asp phosphorelay. A histidine residue in a conserved motif of a histidine kinase is phosphorylated in a reaction that uses ATP. In most cases, it is not known whether the N1 or N3 of the imidazole ring of the histidine side chain is phosphorylated; in CheA, phosphorylation occurs at N3, as shown here. Reversible phosphorelay occurs between His~P and a conserved aspartate residue on a receiver domain, which is present on the same protein or a different protein. Only the side chains of the target histidine and aspartate residues are shown, for simplicity.

biochemical switch to regulate pathway activity. In most cases the ligands themselves are not known, and we know even less about the mechanisms regulating kinase activity in response to ligand binding (Hoch, 2000).

Histidine kinases do not resemble the more familiar protein serine/threonine and tyrosine kinases, either in terms of sequence or structure. Structurally, histidine kinase domains belong to an ancient family of enzymes that use the energy from ATP hydrolysis to drive remodelling reactions, a family that includes DNA topoisomerase II and the chaperone Hsp90 (Bilwes et al., 1999; Stock, 1999; Tanaka et al., 1998). There is limited sequence similarity between histidine kinases and mitochondrial protein serine kinases that phosphorylate large enzyme complexes, such as pyruvate dehydrogenase kinase (Popov et al., 1992, 1993; Thelen et al., 1998).

Given that histidine kinases of phosphorelays phosphorylate only themselves, histidine kinase activities that have been detected on the basis of their phosphorylation of other protein substrates (such as histone H4) are thought to be unrelated to phosphorelay enzymes. Huang et al. (1991) have purified such an activity, p32, from yeast, and this enzyme might represent a fragment of the Arc1p protein, a tRNA synthase cofactor (J. Santos and H. Matthews, personal communication). Besant and Attwood (2000) have detected a similar activity in porcine thymus.

Biochemical analysis of phosphorelay signalling is more problematic than that of conventional protein kinase signalling systems because the phosphorylated histidine and aspartate residues are unstable. A further consequence of this is that it has not been possible to produce anti-phosphohistidine or anti-phosphoaspartate antibodies, but these would be valuable analytical tools.

INVASION OF THE HYBRIDS

Phosphorelays come in essentially two classes. The simpler version, prevalent in bacteria, is often called the two-component system. Here, a histidine kinase and a response regulator directly relay phosphate to one another. An example is the EnvZ-OmpR pathway shown in Fig. 2. A minority of the bacterial phosphorelays use an expanded multistep His-Asp-His-Asp relay mechanism (Appleby et al., 1996). They most commonly employ a histidine kinase that has an attached receiver domain: this is known as a hybrid kinase. Phosphate is first transferred from the histidine residue in the transmitter to the aspartate residue of the attached receiver domain, then to a histidine residue on a histidine phosphotransfer domain (on the same protein or on a separate protein). Finally, the phosphate is relayed from the HPt domain to the receiver domain of a downstream response regulator protein, which results in the output response. An example of such a multistep relay is the BvgS-BvgA system involved in virulence-gene expression in *Bordetella pertussis* (Fig. 2). The bacterial multistep relays display several different architectures that are based on this four-step mechanism. For example, the *Lux* pathway regulating bioluminescence in *V. harveyi* shows the same architecture as the Sln1p-Ypd1p-Ssk1p osmoregulation pathway of *S. cerevisiae* (Fig. 2). However, the *B. subtilis* sporulation pathway does not involve a hybrid kinase, but instead uses four separate proteins for the four-step relay (Fig. 2). The benefit a cell derives from the added sophistication of using a multistep relay as opposed to a two-component system has received much consideration (Appleby et al., 1996). One clear characteristic of multistep relays is increased flexibility of signalling connections between kinases and response regulators, especially in cases in which a separate HPt protein is involved.

Sequence analysis of bacterial phosphorelay genes has shown that there are several subfamilies of both histidine kinase domain and receiver domain. According to the nomenclature of Grebe and Stock (1999), most of the prokaryotic hybrid kinases belong to the HPK1b subfamily,

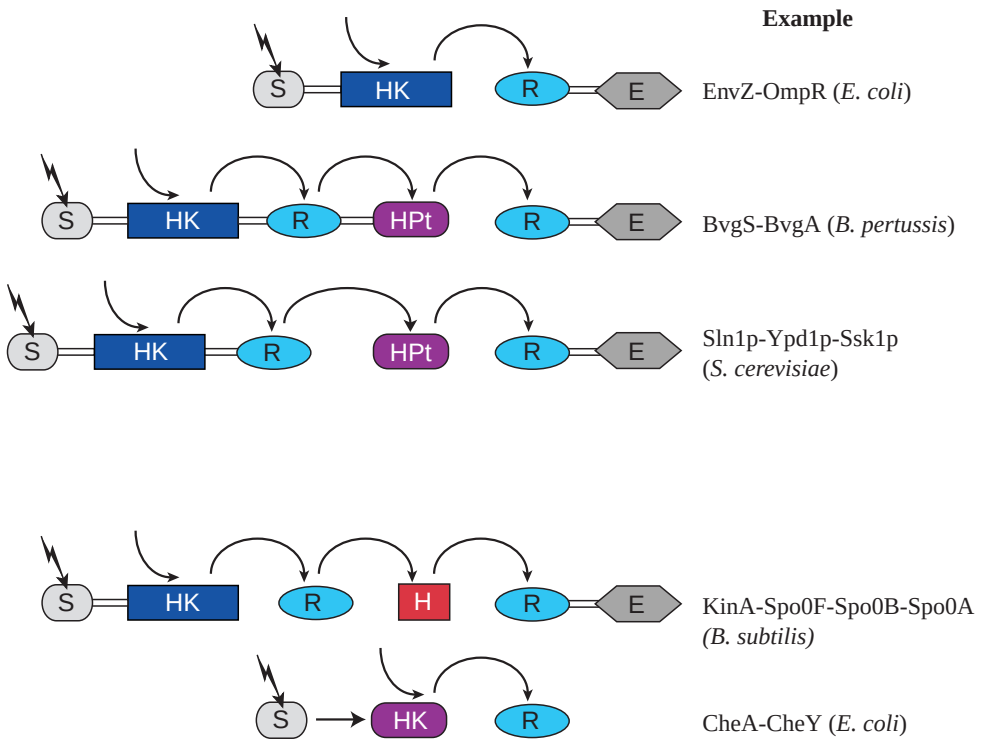

Fig. 2. Phosphorelay architectures. The first three examples illustrate the overall similarities of different phosphorelays. In each, a histidine kinase with an attached sensor domain responds to a signal by changing its net kinase activity. In the EnvZ-OmpR two-component system, phosphate is relayed directly from the phosphorylated HK to the response regulator. In the BvgS-BvgA and Sln1p-Ypd1p-Ssk1p multistep phosphorelays, phosphate is relayed to the attached receiver domain of the hybrid kinase, then to a histidine phosphotransfer domain, and finally to the response regulator. All eukaryotic phosphorelays that have been characterised so far share the architecture of the *S. cerevisiae* osmosensing pathway. The final two examples show important variations on the basic scheme. The *B. subtilis* sporulation pathway was the first multistep phosphorelay pathway to be described, but differs because the intermediate site of histidine phosphorylation, Spo0B, is not a typical HPt domain (Varughese et al., 1998). The chemotaxis system of *E. coli* illustrates other differences. It uses a family of sensory receptors that are detached from the histidine kinase CheA. The kinase itself does not possess a typical substrate domain but instead has an HPt domain that it directly phosphorylates (Zhou et al., 1995). Finally, the response regulator, CheY, consists of only a receiver domain and has no separate output domain. Key: S (sensory domain), HK (histidine kinase), HPt (histidine phosphotransfer domain), H (phosphotransfer domain of Spo0B), R (receiver domain) and E (effector). White rectangles indicate linker regions. The jagged arrow represents a signal that regulates the pathway by increasing or decreasing net histidine kinase activity. Curved arrows represent relay of phosphate between domains. The curved arrow arriving at the HK domain shows transphosphorylation in the dimer complex.

whereas their associated receiver domains belong to the R_B family of receiver domains. Many of the kinases in this subfamily (e.g. BvgS) also contain an HPt domain. Sequence analysis of eukaryotic phosphorelay genes shows that their kinase and receiver modules also belong to the HPK1b and R_B families, respectively. This suggests that all eukaryotic phosphorelay genes evolved from a common ancestral prokaryotic hybrid kinase (which probably also contained an HPt domain; Grebe and Stock, 1999; Pao and Saier, 1997). None of the eukaryotic hybrid kinases discovered so far has an HPt domain; thus this module seems to have become separated during the evolution of the eukaryotic phosphorelay gene family.

PHOSPHORELAYS AND MAP KINASE CASCADES

Stress response pathways in yeasts

The first eukaryotic phosphorelay genes to be discovered were those that encode histidine kinases in *Saccharomyces cerevisiae* (*SLN1*) and *Arabidopsis thaliana* (*ETR1*; Ota and Varshavsky, 1993; Chang et al., 1993). Sln1p is an upstream regulator of the HOG MAP kinase cascade involved in adaptation to osmotic stress. This is the only eukaryotic phosphorelay pathway for which all the components have been biochemically and genetically defined. Because this pathway is well understood, we describe it only briefly. The transmembrane histidine kinase Sln1p is active under normal growth conditions (Maeda et al., 1994). It autophosphorylates and transfers phosphate (via its receiver domain) to the HPt protein, Ypd1p, and subsequently to the response regulator Ssk1p (Fig. 2; Posas et al., 1996). Phosphorylation of Ssk1p prevents it from activating the Ssk2p and Ssk22p MAP kinase kinase kinases that activate the downstream components in the HOG pathway (Posas and Saito, 1998). Hyperosmolarity causes inhibition of Sln1p, allowing unphosphorylated Ssk1p to accumulate and the HOG pathway to be activated (Fig. 3). *SLN1* is essential for normal growth, given that constitutive activity of the HOG pathway is lethal (Maeda et al., 1994). The mechanism by which Sln1p senses osmotic changes is not known, but it can respond to osmotic stresses that are imposed extracellularly or generated intracellularly (Tao et al., 1999); thus its precise conformation in the membrane must be subject to change.

Recent work has revealed that *Schizosaccharomyces pombe* operates a strikingly similar system that responds to peroxide stress rather than hypertonic stress (Fig. 3). Here, the response regulator protein, Mcs4p, an Ssk1p homologue (Cottarel, 1997; Shieh et al., 1997; Shiozaki et al., 1997), controls the activity of the Wak1p-Wis1p-Sty1p stress-activated MAP kinase cascade. A phosphorelay pathway is initiated by two *S. pombe* histidine kinases, Mak2p and Mak3p, (for Mcs4p-activating kinases; J. Millar, personal communication). Genetic evidence suggests that Mak2p and Mak3p operate as a heterodimer.

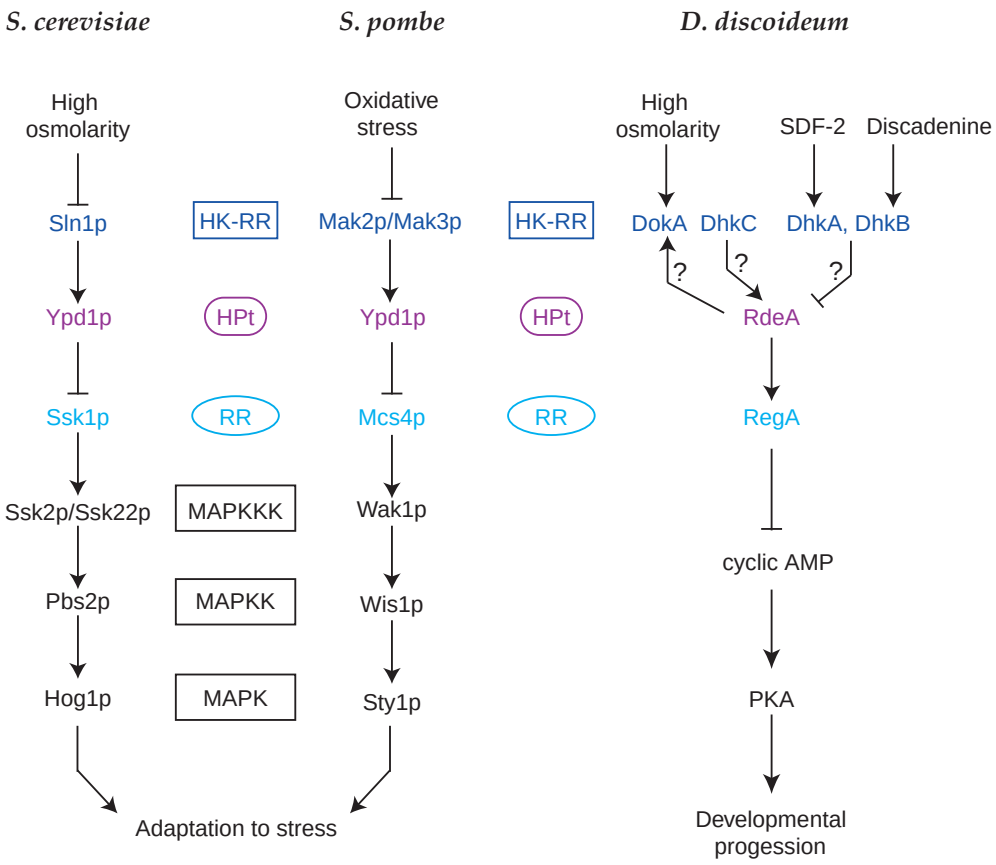

Fig. 3. Phosphorelay-controlled signal transduction pathways. The well understood *S. cerevisiae* phosphorelay controlling the HOG osmotic adaptation pathway is shown beside a proposed homologous phosphorelay from *S. pombe* that responds to oxidative stress (J. Millar, personal communication). On the basis of genetic evidence, the unphosphorylated form of Mcs4p is thought to be active (as with Ssk1p), but biochemical confirmation is awaited. A different kind of pathway is illustrated by the RdeA-RegA phosphorelay, which controls cAMP levels in *D. discoideum*. A common feature of these three eukaryotic phosphorelays is their control of 'conventional' protein kinase signalling pathways. HK (histidine kinase), HPt (histidine phosphotransfer protein), RR (response regulator), MAPK (mitogen activated protein kinase).

They each contain a single PAS and PAC motif, and a GAF domain, which are potentially involved in sensing oxidative stress. Mak2p and Mak3p signal through *S. pombe* Ypd1p (also called Mpr1p) to control phosphorylation of Mcs4p on its receiver domain (J. Millar, personal communication; Nguyen et al., 2000). *Mcs4+* is required for the activation of the MAP kinase Sty1p in response to many other stresses, such as hyperosmolarity, UV light, presence of the protein synthesis inhibitor anisomycin, and temperature shock, but these responses do not involve Mcs4p-receiver-domain phosphorylation (Shieh et al., 1997; Shiozaki et al., 1997).

In *S. cerevisiae* there is only one further response regulator, Skn7p, whose regulation shows similarities to that of Mcs4p in *S. pombe*. Skn7p is a heat-shock-like DNA-binding transcription factor that functions in several processes, including cell wall maintenance, cell cycle progression, osmotic adaptation, and the response to oxidative stress (Bouquin et al., 1999; Brown et al., 1994; Li et al., 1998; Morgan et al., 1997). Many of these functions require phosphorylation of Asp427 in the receiver domain, and it has been shown that Skn7p receives this phosphate from Sln1p, via Ypd1p (Li et al., 1998). The oxidative stress response, however, does not require *SLN1* or *YPD1*, and does not involve phosphorylation of D427 (Li et al., 1998; Morgan et al., 1997). The mechanism of activation of Skn7p by oxidative stress is not known, but could involve phosphorylation of Skn7p on serine/threonine and tyrosine residues: it is known that Skn7p isolated from cells is a phosphoprotein even in the absence of D427 phosphorylation (Brown et al., 1994).

In *S. pombe* a second response regulator, Prr1p, is closely related to Skn7p. Prr1p is also involved in adaptation to oxidative stress (Ohmiya et al., 1999) but, unlike Mcs4p, does not act downstream of Mak2p or Mak3p. Instead, it might be regulated by a third *S. pombe* histidine kinase, Mak1p (J. Millar, personal communication), since both *mak1+* and *prr1+* are required for peroxide-induced catalase expression. Because a *ypd1–* (*mpr1–*) strain is still able to induce catalase expression in response to peroxide stress (Ohmiya et al., 1999), any putative Mak1p-Prr1p phosphorelay might not include Ypd1p.

Hormone signalling in plants

The hormone ethylene regulates many plant responses, the most familiar being fruit ripening (Chang, 1996). In *Arabidopsis thaliana* ethylene is sensed by a family of five receptor histidine kinases (Hua and Meyerowitz, 1998; Gamble et al., 1998). The receptors are constitutively active in the absence of their ligand, and are inhibited by ethylene binding (possibly through disruption of receptor dimers; Hua and Meyerowitz, 1998). This is the opposite of the normal paradigm for receptor signalling, whereby ligand binding induces receptor dimerisation and activation. *Arabidopsis* plants that have dominant (gain-of-function) mutations in a single ethylene receptor gene are ethylene insensitive: their signalling activity cannot be turned off. Conversely, plants that have single recessive loss-of-function mutations have no phenotypic defect (because other receptors can compensate). The *etr1- etr2- ein4- ers2-* quadruple mutant, in which four of the five ethylene receptor genes are disrupted, exhibits a constitutive response in the absence of the hormone – i.e.

ethylene is no longer required for repression of receptor activity (because this is already low; Hua and Meyerowitz, 1998).

The Ctr1 Raf-like protein kinase is a negative regulator of ethylene responses and acts downstream of the receptors (Chang, 1996). In the absence of hormone, receptor activity keeps Ctr1 active and ethylene responses repressed. It is not known whether the ethylene signalling pathway resembles the yeast multistep phosphorelays discussed above. Clark et al. (1998) have reported that Ctr1 can bind directly to ethylene receptors, which suggests that direct communication occurs. Although it is anticipated that Ctr1 would regulate a MAP kinase module, no such signalling connections have been established in the ethylene response pathway.

A second class of plant hormone, the cytokinins, signal via the *Arabidopsis* CKI1 (and possibly CKI2) receptor histidine kinases (Kakimoto, 1996; Urao et al., 2000). The signalling pathways activated by cytokinin receptors might involve several *Arabidopsis* response regulators whose expression is induced by hormone treatment. The fourteen *Arabidopsis* response regulators that have been named so far fall into two families. Type A response regulators have short C-termini and no clear output domain, whereas type B response regulators have long C-terminal extensions that appear to be DNA-binding domains (D'Agostino and Kieber, 1999; Urao et al., 2000). The type A response regulators induced by cytokinin are able to participate in in vitro phosphotransfer reactions with members of the *Arabidopsis* HPt family, which suggests that cytokinin signal transduction involves a multistep phosphorelay (D'Agostino and Kieber, 1999; Urao et al., 2000). The same might be the case for a recently isolated osmotic response histidine kinase, ATHK1 (Urao et al., 1999). Direct evidence concerning the role of plant HPt homologues in hormone signalling is still awaited.

An important evolutionary concern involves the phytochromes, which are plant photosensory receptors. Sequence analysis shows that phytochromes are derived from ancestral cyanobacterial genes that encode histidine kinases (Yeh et al., 1997). Plant phytochromes are thought to be light-regulated protein serine kinases (Yeh and Lagarias, 1998), although debate has raged over whether they do have intrinsic protein kinase activity (Elich and Chory, 1997). One point is clear: plant phytochromes have diverged sufficiently from the cyanobacterial genes no longer to encode functional histidine kinases.

DICTYOSTELIUM: THE REMARKABLE AMOEBA

Clearly, among eukaryotes, plants make the greatest use of phosphorelay signalling (see Table 1). *Dictyostelium discoideum*, a social amoeba, provides the most striking example of eukaryotic phosphorelay signalling outside of plants. *Dictyostelium* genes encoding phosphorelay components were first discovered in genetic screens for morphological mutants and by homology cloning (Schuster et al., 1996; Shaulsky et al., 1996; Wang et al., 1996). Genome sequencing is now revealing the extent of this gene family: currently, 20 phosphorelay genes have been identified by the *Dictyostelium* genome project (Table 1), but only one phosphorelay pathway has been studied biochemically. *Dictyostelium* mutants of phosphorelay genes studied so far have defects in the developmental cycle. Because the developmental programme has been extensively studied, and since phosphorelay signalling appears to be a major regulator of the programme, this is a very attractive system for analysis (Aubry and Firtel, 1999).

During *Dictyostelium* development, individual amoebae move chemotactically towards cyclic AMP, which is released by the cells themselves, and aggregate into a mound (Parent and Devreotes, 1996). Cell differentiation and morphogenesis follow, giving rise to a fruiting body of terminally differentiated stalk and spore cells. The rate of development is controlled largely by protein kinase A and is thus dependent on the concentration of intracellular cAMP. Two response regulators, AcrA and RegA, control the synthesis and breakdown, respectively, of cAMP(see below).

The RdeA-RegA phosphorelay controls cAMP breakdown

The only *Dictyostelium* phosphorelay to have been studied biochemically is the RdeA-RegA phosphorelay that determines the rate of cAMP hydrolysis (see Fig. 3; Chang et al., 1998; Thomason et al., 1998). RegA is a very rare example of a response regulator that has an enzymatic output, this being a C-terminal cAMP phosphodiesterase domain (Shaulsky et al., 1996, 1998; Thomason et al., 1998). Phosphodiesterase activity is strongly stimulated by phosphorylation of RegA on its receiver domain, through phosphotransfer from the HPt protein RdeA (Thomason et al., 1999b). Control of RegA activity is essential for coordination of morphogenesis and terminal differentiation during late development, but is probably important throughout the developmental cycle (Thomason et al., 1999a).

It is possible that the RdeA-RegA phosphorelay is a target for many upstream histidine kinases. At least 15 histidine kinases are present in *Dictyostelium* (Table 1), four of which – DhkA, DhkB, DhkC and DokA – have been studied in some detail. *DhkC*-null strains (Singleton et al., 1998) have rapid early development, which suggests that cAMP levels are high in these strains; thus DhkC might be an activator of RegA during this stage. Conversely, DhkB might be an inhibitor of RegA. DhkB acts very late in development to control germination of spores (Zinda and Singleton, 1998) and is part of the reception mechanism for the spore-germination inhibitor discadenine (which, like plant cytokinins, is an adenine derivative). In contrast to earlier stages of development, spore germination is inhibited by high cAMP levels (Virdy et al., 1999). Since *dhkB*-null mutants cannot delay spore germination appropriately, and have low cAMP levels, DhkB might inhibit RegA – through an unknown mechanism. DokA, which controls osmosensing in *Dictyostelium* amoebae and possibly also in developing spores (Schuster et al., 1996), might also inhibit RegA. In *dokA*-null mutants the developing spores appear incapable of adapting to the high osmotic environment of the spore head. There is evidence that DokA communicates with RdeA (S. Schuster, personal communication); thus, DokA might inhibit RegA by withdrawing phosphate from the RdeA-RegA module (see Fig. 3).

DhkA, the best-studied histidine kinase in *Dictyostelium* (Wang et al., 1996), is a transmembrane protein believed to be the receptor for a signalling peptide, SDF-2, that is released by pre-stalk cells to induce the maturation of pre-spore cells

Table 1. Phosphorelay genes (known and novel) in eukaryotes

Organism	Kinase (input signal)	HPt	Response regulator (activity)
S. cerevisiae	**Sln1p** (osmolarity)	**Ypd1p**	**Ssk1p** (MAPKKK regulator) **Skn7p** (Heat shock-like TF)
S. pombe	*Mak2p (oxidative stress) *Mak3p (oxidative stress) *Mak1p (oxidative stress)	**Ypd1p/Mpr1p**	**Mcs4p** (MAPKKK regulator) **Prr1p** (Heat shock-like TF)
C. albicans	CaNIK-1 CaSLN-1 (osmolarity?) CaHK-1	*CaYPD1	CaSSK1
D. discoideum	DokA (osmolarity) DhkA (SDF-2 peptide) DhkB (discadenine) DhkC (ammonia?) DhkD *SmbA *DdNIK-1 *EST sequences (4) *Genomic sequences (4)	**RdeA**	**RegA** (cAMP PDE) AcrA (adenylyl cyclase) *DdRR3 *DdRR4
A. thaliana	ETR1 ETR2 EIN4 ERS1 ERS2 **CKI1** (cytokinin) **ATHK1** *CKI2 (cytokinin?) *Genomic sequences (3)	**ATHP 1-3** *Genomic (7)	Type B RRs (7) **Type A RRs (7)** *Genomic sequences (8)
N. crassa	NIK-1 *NIK-2		
A. nidulans	*NIK-1		

Genes that encode cognate signalling partners in each organism are shown in red. Genes encoding proteins for which there is some biochemical evidence of interaction are shown in blue. Direct evidence for any other connections is awaited; these are shown in black. Many more plant phosphorelay genes are known but these are currently restricted to homologues of the *Arabidopsis* genes (see Urao et al. (2000) for more information). Unpublished sequences are indicated by an asterisk (*). The accession numbers for the *S. pombe* histidine kinase genes are as follows: *mak2+* (AL034352), *mak3+* (AL031543), *mak1+* (AL157734) (J. Millar, personal communication). *S. pombe ypd1+* and *mpr1+* are the same gene (Nguyen et al., 2000) (J. Millar). Information sources for other unpublished genes: *N. crassa NIK-2* (Alex et al., 1996); *A. nidulans NIK-1* is published as a partial sequence (Alex et al., 1998); *C. albicans YPD1* (Calera et al., 2000). The five *D. discoideum* ESTs were identified from a cDNA database located at http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html and belong to the following families of clones: SSI667, SLK607, SSK767, SLC110, SSA688 (*smbA*). The *Dictyostelium* genomic sequences (DdNIK-1, the four kinases arbitrarily named Kin12-Kin15, and the two response regulators DdRR3 and DdRR4) were identified from the *D. discoideum* genome databases located at http://genome.imb-jena.de/dictyostelium, http://www.sanger.ac.uk/Projects/D_discoideum/ and http://glamdring.ucsd.edu/others/dsmith/dictydb.html#F . The Type A *Arabidopsis* response regulators are ARR3-ARR9; the Type B regulators are ARR1, ARR2, and ARR10-ARR14 (D'Agostino and Kieber, 1999; Urao et al., 2000).

(Anjard et al., 1998a,b; Wang et al., 1999). *DhkA*-null mutants do not respond to SDF-2 and make very few spores during development. The mechanism of action of SDF-2/DhkA is not entirely clear, but may involve the inhibition of RegA (Anjard et al., 1998b; Wang et al., 1999).

AcrA controls cAMP production

A second putative response regulator in *Dictyostelium*, the adenylyl cyclase AcrA, regulates cAMP production rather than hydrolysis. *Dictyostelium* has at least three adenylyl cyclase enzymes, of which AcrA is the most recently identified. It was first detected biochemically – in cells in which *regA* was inactivated by genetic disruption or pharmacological inhibition – and was given the name ACB (Kim et al., 1998). A candidate for the gene encoding this enzyme (*acrA*) was identified in a genetic screen for mutants in which fruiting-body construction is defective. *AcrA* encodes an adenylyl cyclase that also has two other domains: a receiver domain, and a diverged histidine

kinase domain (Soderbom et al., 1999). AcrA is closely related to the CyaC enzymes from the cyanobacteria *Spirulina* and *Anabaena*, though these adenylyl cyclases also have an intact histidine kinase domain (Kasahara et al., 1997; Katayama and Ohmori, 1997). Recently Kasahara and Ohmori, (1999) showed that *Spirulina* CyaC is activated by phosphorylation of its receiver domain (via phosphotransfer from the histidine kinase domain). Any putative regulation of the *Dictyostelium* AcrA enzyme has yet to be demonstrated. *AcrA*-null strains develop normally until the time of fruiting-body formation, but then make fragile stalks and very few spores (Soderbom et al., 1999). This is very similar to the phenotype of *dhkA*-null mutants.

Further histidine kinase and response regulator genes that have been identified in the *Dictyostelium* genome project can be analysed by reverse genetics (gene disruption by homologous recombination) and molecular genetics. Using this approach, we have recently found that a histidine kinase, which we have named *Sombrero* (*smbA*), is required for later stages of morphogenesis. The gene is expressed at a time during development when migrating slugs begin to round up prior to fruiting-body construction, and *smbA* mutants cannot progress beyond this stage (P. Thomason and R. Kay, unpublished). The signalling pathway involved is not presently known.

SIGNALLING LOGIC AND CONNECTIONS

Phosphorelay components must interact with each other in specific combinations, despite possessing common structural elements. For the simpler phosphorelays (i.e. the two-component systems), a histidine kinase and a response regulator often form a cognate pair. However, greater complexity can exist – for instance, the histidine kinase of bacterial chemotaxis, CheA, phosphorylates both CheY and CheB response regulators (Bourret et al., 1991). Additionally, multiple histidine kinases can target a single response regulator (e.g. in *Bacillus subtilis*, KinA, KinB and KinC all phosphorylate Spo0F; Burbulys et al., 1991).

Eukaryotic phosphorelays may be specialised for their extensive use of separate HPt proteins (rather than HPt domains attached to hybrid kinases). Phosphorelays that employ a separate HPt protein appear to be a small minority of the multistep relays in prokaryotes (Freeman and Bassler, 1999), whereas in eukaryotes this set-up seems to predominate. Evidence suggests that these separate HPt proteins undertake many interactions. For example, in *S. cerevisiae*, the only HPt protein present, Ypd1p, participates in phosphotransfer with three receiver domains: those of Sln1p, Ssk1p and Skn7p (Posas et al., 1996; Li et al., 1998). In vitro, Ypd1p will also communicate with the bacterial response regulator CheY (Janiak-Spens et al., 1999). The crystal structures of Ypd1p and of other HPt domains reveal an antiparallel four-helix bundle in which the acceptor histidine residue is located midway up one helix and is highly accessible (Song et al., 1999; Xu and West, 1999). It seems only logical that this active site will be accessible to more receiver domains when it is located on a separate protein.

The *Dictyostelium* RdeA protein can also communicate with heterologous signalling proteins in vitro (e.g. it is a substrate for the catalytic domain of CheA; Thomason et al., 1999b), which suggests that it takes part in multiple interactions in the cell. This is especially pertinent given that RdeA is the only known HPt domain in *Dictyostelium*. Completion of the genome sequence within the next year will reveal the full complement of phosphorelay genes in *Dictyostelium*, and, although further HPt and response regulator genes will undoubtedly be found, it is very unlikely that their number will approach that of the histidine kinases. Interestingly, *Dictyostelium* uses an 'excess' of kinases, whereas *Arabidopsis*, which has at least 10 histidine kinases and 22 response regulators (Urao et al., 2000), has an 'excess' of response regulators. On a superficial level this suggests that the *Dictyostelium* receptor kinases funnel in to just a few signalling pathways, whereas *Arabidopsis* receptor kinases might activate divergent downstream signalling events (initial studies of cytokinin signalling support this notion). A rationalisation for this is that the pathways controlled by *Dictyostelium* phosphorelays (e.g. the cAMP/PKA signalling pathway) themselves have many targets.

How do phosphorelays interact with the 'normal' eukaryotic signalling systems? Information on this is still quite scant, but there are a few examples. The *S. cerevisiae* HOG MAP kinase pathway described above integrates information not only from the Sln1p-Ypd1p-Ssk1p phosphorelay (see Fig. 3) but also from a second osmosensor, Sho1p. Sho1p is a transmembrane protein that has an intracellular SH3 domain required for the activation of the Ste11p MAP kinase kinase kinase (Posas and Saito, 1997) that subsequently activates the Pbs2p MAP kinase kinase, the common entry point for the two osmosensors. These two systems therefore use entirely distinct mechanisms (the first 'novel', the second 'conventional') to control a common MAP kinase module. The Skn7p response regulator interacts genetically or biochemically with the small GTPases Rho1p and Cdc42p (Alberts et al., 1998), and the cell cycle transcription factor Mbp1p (Bouquin et al., 1999). It is also phosphorylated at residues other than the receiver domain aspartate in vivo, which suggests that it is regulated by protein kinases (Brown et al., 1994). *Dictyostelium* RegA might be similar, since it is multiply phosphorylated in vivo (P. Thomason and R. Kay, unpublished).

PHOSPHORELAYS AND ANTI-MICROBIAL THERAPY

Not surprisingly, phosphorelays are attracting attention as potential therapeutic targets. Since the pathways are apparently absent from the animal kingdom, they seem to be eminently suitable. Furthermore, some phosphorelays are specifically involved with the establishment of virulence – for example, in bacteria such as *Bordetella pertussis* (Beier et al., 1995; Uhl and Miller, 1996) – whereas others are associated with antibiotic resistance mechanisms (Arthur et al., 1992; Guenzi et al., 1994; Hakenbeck et al., 1999). Little progress has so far been made in developing suitable compounds. In two studies, compounds that inhibited in vitro phosphotransfer assays were found to have antibacterial and antifungal activity (Deschennes et al., 1999; Hilliard et al., 1999), but sadly this was not related to phosphorelay inhibition and was probably due to nonspecific membrane damage.

The best indications of target validity have come from gene-

knockout work. For example, in studies of the bacterium *Streptococcus pneumoniae*, which causes pneumonia, meningitis and other invasive infections, single-gene mutations were made in each of the 14 response regulators (or in kinase-regulator pairs) of this organism (Lange et al., 1999; Throup et al., 2000). Roughly half of the mutants had reduced virulence in a mouse respiratory-tract infection assay (Throup et al., 2000) but not in a bacteriaemic infection assay (Lange et al., 1999). The importance of the signalling systems thus depends on the environment to which the organism must adapt.

Similar studies have begun in fungi. Dimorphism (the yeast-hyphal transition) is one of several virulence determinants in the opportunistic pathogen *Candida albicans* (Corner and Magee, 1997). Single mutants of the histidine kinases *CaNIK-1*, *CaSLN-1* and *CaHK1* have reduced hyphal development on solid medium (Alex et al., 1998; Calera and Calderone, 1999; Srikantha et al., 1998; Yamada-Okabe et al., 1999). In a mouse systemic infection assay, all these mutants have reduced virulence, with the *CaHK1* mutant being the most attenuated (Yamada-Okabe et al., 1999). A mutant of *CaSSK1* (the only identified response regulator in *C. albicans*, and not a functional homologue of *S. cerevisiae SSK1*) has altered hyphal development and is completely avirulent in a mouse model of infection (Calera et al., 2000). Interestingly, a *CaHOG1*-null *C. albicans* strain has certain similarities to the *CaSSK1* mutant (Alonso-Monge et al., 1999). Many genes regulate *C. albicans* morphogenesis (Brown and Gow, 1999), including those that encode the two transcription factors Cph1 and Efg1. A MAP kinase pathway regulates Cph1, but this currently does not include CaHog1. Another transcription factor, Tup1, which is involved in repression of hyphal development, has been proposed to be a target of CaHog1 (Alonso-Monge et al., 1999).

Homologues of *CaNIK-1* are present in several other species, including the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* (Alex et al., 1996), the filamentous bacterium *Streptomyces coelicolor*, and *Dictyostelium discoideum*. *NIK-1* mutants of *N. crassa* have reduced hyphal development, which might be a consequence of impaired cell wall maintenance. The conservation of Nik-1 between species is remarkable: the protein is conserved over its entire length, including the novel N-terminal 90-residue repeats that might couple it to a membrane receptor. A role for Nik-1 in virulence of filamentous fungi has yet to be investigated.

CONCLUSIONS

Much of our understanding of eukaryotic phosphorelay signal transduction has come from studies of stress responses in yeast, hormone signalling in *Arabidopsis*, and cAMP metabolism in *Dictyostelium*. Deciphering the signalling connections between phosphorelay components, especially in plants and *Dictyostelium*, which have large phosphorelay families, is a major challenge. Understanding the basis of the specificity (or flexibility) of these interactions will require extensive genetic and biochemical analyses. Intersections between phosphorelays and other signalling pathways remain to be fully dissected. Finally, just as in prokaryotes, the nature of the signals that control these pathways in eukaryotes remains largely unknown but holds the key to understanding of their biological function.

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