

# Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors

(CheY/CheB/acetyl phosphate/carbamoyl phosphate/phosphoramidate)

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**ABSTRACT** Bacterial motility and gene expression are controlled by a family of phosphorylated response regulators whose activities are modulated by an associated family of protein-histidine kinases. In chemotaxis there are two response regulators, CheY and CheB, that receive phosphoryl groups from the histidine kinase, CheA. Here we show that the response regulators catalyze their own phosphorylation in that both CheY and CheB can be phosphorylated in the complete absence of any auxiliary protein. Both CheY and CheB use the N-phosphoryl group in phosphoramidate ( $\text{NH}_2\text{PO}_3^{2-}$ ) as a phospho-donor. This enzymatic activity probably reflects the general ability of response regulators to accept phosphoryl groups from phosphohistidines in their associated kinases. It provides a general method for the study of activated response regulators in the absence of kinase proteins. CheY can also use intermediary metabolites such as acetyl phosphate and carbamoyl phosphate as phospho-donors. These reactions may provide a mechanism to modulate cell behavior in response to altered metabolic states.

In bacteria, a family of phosphorylated response regulators controls gene expression and motility in response to environmental signals (for reviews see refs. 1–3). These proteins are generally composed of a conserved N-terminal regulatory domain of  $\approx 120$  amino acids linked to variable C-terminal effector domains. The response regulator that controls motility in chemotaxis, CheY, is an exception in that it is composed solely of the regulatory domain. It has an  $\alpha/\beta$  structure with a five-stranded parallel  $\beta$ -sheet surrounded by five  $\alpha$ -helices (4). CheY is phosphorylated at Asp-57, a residue that is conserved in all homologous response regulator proteins (5). Two additional highly conserved residues, Asp-12 and Asp-13, bind a  $\text{Mg}^{2+}$  ion that is essential for phosphorylation (6, 7). CheY, like many other response regulators, has an associated autophosphatase activity; phospho-CheY has a half-life of  $\approx 10$  sec. Phosphatase activity is enhanced by an auxiliary regulatory protein, CheZ (8).

The activity of response regulators is controlled by a family of histidine kinases that are autophosphorylated in the presence of ATP (1–3). The phosphotransfer mechanism involves the intermediate formation of a phosphohistidine residue in the kinase. In chemotaxis, the rate of phosphorylation of the histidine residue in the kinase, CheA, is stimulated by membrane chemoreceptor proteins (9, 10). The phosphoryl group is then rapidly transferred to CheY to control motility. Another chemotaxis response regulator, CheB, also accepts phosphoryl groups from CheA. CheB provides a feedback adaptation mechanism. Phosphorylation of its N-terminal regulatory domain stimulates a C-terminal catalytic activity that modifies the chemoreceptors to attenuate CheA kinase activity (9, 11).

Although in many cases a specific kinase has been implicated in the regulation of a given response regulator, considerable cross specificity has also been observed (12–14). The phenotypes of kinase mutants have indicated that response regulators can be phosphorylated in the absence of their cognate kinases (15–20). Here we show that CheY and CheB are enzymes that catalyze their own phosphorylation using low molecular weight phospho-donors. Thus, the enzymology of aspartate phosphorylation is an inherent property of the response regulators that can occur independently of any other protein.

## MATERIALS AND METHODS

**Materials.** The ammonium and potassium salts of phosphoramidate were synthesized by the method of Sheridan *et al.* (21). Acetyl phosphate and carbamoyl phosphate were purchased from Sigma. *S*-Adenosyl-L-[methyl- $^3\text{H}$ ]methionine (75 Ci/mmol; 1 Ci = 37 GBq) and [ $^{32}\text{P}$ ]phosphoric acid (9000 Ci/mmol) were purchased from New England Nuclear/DuPont. Dilitium acetyl [ $^{32}\text{P}$ ]phosphate was prepared by the method of Stadtman (22).

**Proteins.** Wild-type and mutant CheY proteins as well as CheZ and CheB were purified as described (23–25). Protein purity was estimated to be  $>95\%$  on the basis of SDS/PAGE. [ $^3\text{H}$ ]Methyl-labeled Tar receptor in *Escherichia coli* membranes was prepared as described (25).

**Fluorescence Measurements.** Steady-state fluorescence measurements were obtained with a Perkin-Elmer MPF66 spectrofluorometer interfaced to a Perkin-Elmer 7500 computer. The temperature of the sample was kept at  $20^\circ\text{C}$  with a thermostatted metallic cell holder to a precision of  $\pm 0.1^\circ\text{C}$ . The excitation wavelength was set at 295 nm for selective excitation of tryptophan, and in all cases, the solution absorbance was  $<0.1$  at the excitation wavelength. All additions of reagents were done with volumes that resulted in no more than a 2% dilution.

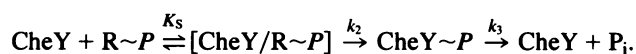
**Measurements of Phospho-Donor Hydrolysis.** Various proteins (6  $\mu\text{g}$ ) were incubated in 10  $\mu\text{l}$  of a buffer containing 100 mM Tris-HCl (pH 7.0), 5 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, and 10 mM acetyl [ $^{32}\text{P}$ ]phosphate. At the indicated times, 2- $\mu\text{l}$  samples were added to 10  $\mu\text{l}$  of 100 mM Tris-HCl, pH 7.0/10 mM EDTA/1% SDS and immediately placed on ice. Aliquots of 1  $\mu\text{l}$  were analyzed by chromatography on PEI-cellulose sheets in 1 M LiCl/1% (vol/vol) acetic acid. After autoradiography, the spots corresponding to acetyl phosphate were excised and radioactivity was measured by liquid scintillation spectrometry.

The hydrolysis of phosphoramidate in the presence and absence of CheY or CheB was monitored by  $^{31}\text{P}$  NMR at 101 MHz. Reactions were initiated by addition of phosphoramidate immediately before samples were placed in the NMR spectrometer.

## RESULTS

**Phosphorylation of CheY by Low Molecular Weight Phospho Donors.** CheY has a single tryptophan, Trp-58, which is adjacent to the site of phosphorylation, Asp-57 (23). Intrinsic fluorescence was used as a probe for CheY phosphorylation by low molecular weight phospho-donors. When  $Mg^{2+}$  was present, acetyl phosphate, carbamoyl phosphate, and phosphoramidate caused significant fluorescence quenching. The data shown in Fig. 1 for the reaction of CheY with acetyl phosphate are typical of the reactions with all three of the phospho-donors studied. In all cases, titration of CheY with phospho-donor (events A and B, Fig. 1) caused a decrease in fluorescence intensity over a time course of 30–60 sec until a new steady-state value was obtained. Additions of catalytic amounts of CheZ reversed the quenching (events C, D, and E); this is consistent with the fact that CheZ increases the rate of phospho-CheY hydrolysis. The effect of CheZ provides strong evidence that the phospho-donors are phosphorylating CheY at its normal active site. Furthermore, removal of the phospho-donor reverses the fluorescence quenching with rates that are consistent with those previously reported for the autocatalyzed dephosphorylation of CheY (6). Acyl phosphates and phosphoramidate had no significant effect on the fluorescence of CheY proteins mutated at active-site aspartate residues (CheYD13N, CheYD57N).

CheY may be considered as a phospho-donor (R~P) phosphatase with phospho-CheY an enzyme intermediate:



This model can be used to fit the observed fluorescence of CheY in the presence of  $Mg^{2+}$  and varying phospho-donor concentrations (Fig. 2). The reciprocal of the slope of the line for each phospho-donor in Fig. 2 corresponds to the  $K_m$  of the CheY phosphatase for that donor, where  $K_m = K_S k_3 / k_2$  in the above scheme. The  $K_m$  values for acetyl phosphate, carbamoyl phosphate, and phosphoramidate were 0.70 mM, 1.2 mM, and 1.8 mM, respectively. Intracellular concentrations of acetyl phosphate in excess of 1 mM have been reported (26). Thus, acetyl phosphate may contribute significantly to phosphorylation of CheY *in vivo*.

Phosphorylation of CheY by acetyl phosphate was confirmed using acetyl [ $^{32}\text{P}$ ]phosphate (Fig. 3, lane 2). CheY was not phosphorylated in the absence of  $Mg^{2+}$  (lane 1) or when the active-site Asp-57 was changed to Asn (CheYD57N) (lane

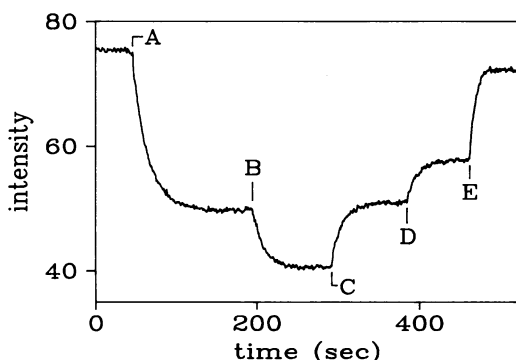


FIG. 1. Fluorescence intensity as a function of time for the reaction of CheY with acetyl phosphate and CheZ. The reactions were carried out with 4.0  $\mu\text{M}$  CheY and 8.8 mM  $MgCl_2$  at 20°C in 100 mM Tris-HCl (pH 7.0). Excitation wavelength was 295 nm; fluorescence intensity was monitored at 346 nm. At the indicated times, reactants were added to give the following concentrations: 1.2 mM acetyl phosphate (A), 2.4 mM acetyl phosphate (B), 26 nM CheZ (C), 39 nM CheZ (D), and 92 nM CheZ (E). The final steady-state fluorescence intensity after event E is equivalent to the initial fluorescence when dilution effects are considered.

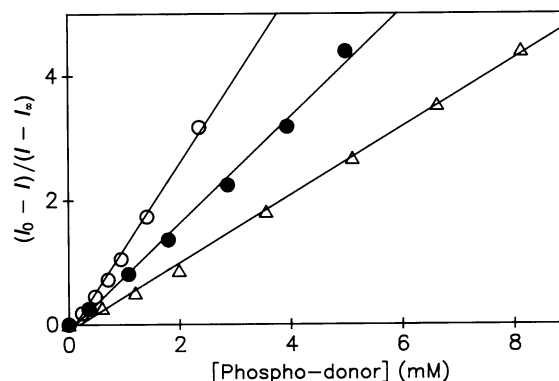


FIG. 2.  $(I_0 - I)/(I - I_\infty)$  versus phospho-donor concentration [R~P], for the reaction of CheY with acetyl phosphate (○), carbamoyl phosphate (●), or phosphoramidate (△). Experimental conditions are the same as in Fig. 1. If it is assumed that the observed quenching is a direct effect of the reduced quantum yield of phospho-CheY relative to that of CheY, the steady-state fluorescence at a given concentration of phospho-donor may be related to the kinetic parameters of the reaction scheme proposed in the text, where  $(I_0 - I)/(I - I_\infty) = ([R\sim P]k_2)/(k_3K_S)$ ;  $I_0$  is fluorescence intensity of the CheY/ $Mg^{2+}$  complex in the absence of R~P,  $I_\infty$  is the fluorescence intensity at saturating R~P, and  $I$  is the steady-state fluorescence intensity at indicated values of [R~P].

3). Proteins such as bovine serum albumin or CheW (lanes 5 and 6) were not significantly phosphorylated by acetyl [ $^{32}\text{P}$ ]phosphate. Surprisingly, CheB, a response regulator homolog of CheY that also accepts a phosphoryl group from CheA, was not phosphorylated by acetyl phosphate (lane 4). The lack of phosphorylation of serum albumin and CheW and the specificity of phospho-donors for CheB emphasize that the observed phosphorylations are enzymatic and are not spontaneous reactions of highly reactive phosphorylating agents.

Several other compounds were examined for their ability to react with CheY, including  $P_i$ ,  $PP_i$ , ATP, and creatine phosphate. In no case was phospho-CheY detected. Preliminary results indicate that phosphoramidates such as phosphoimidazole also serve as effective phospho-donors.

**Phosphatase Activities of CheY and CheB.** Rates of hydrolysis of acetyl phosphate and phosphoramidate were measured in the presence and absence of CheY and CheB (Fig. 4). CheY catalyzed the hydrolysis of both of these phospho-donors, whereas the effect of CheB was specific for phosphoramidate.

**CheB Activation by Phosphoramidate.** Phosphorylation of CheB by the chemotaxis kinase, CheA, causes a dramatic

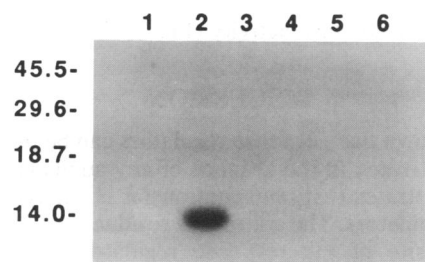


FIG. 3. Phosphorylation of CheY by acetyl [ $^{32}\text{P}$ ]phosphate. Various proteins (450 pmol) were incubated for 1 min at 25°C in 50 mM Tris-HCl, pH 7.0/5 mM  $MgCl_2$ /1 mM dithiothreitol/10 mM acetyl [ $^{32}\text{P}$ ]phosphate (8.5 mCi/mmol) in a final volume of 15  $\mu\text{l}$ . Reactions were stopped by the addition of 5  $\mu\text{l}$  of 4 $\times$  SDS/PAGE loading buffer containing 10 mM EDTA and proteins were separated in an SDS/15% polyacrylamide gel. Lanes: 1, CheY plus 5 mM EDTA; 2, CheY; 3, CheYD57N; 4, CheB; 5, CheW; 6, bovine serum albumin. Molecular size markers (kDa) are at left.

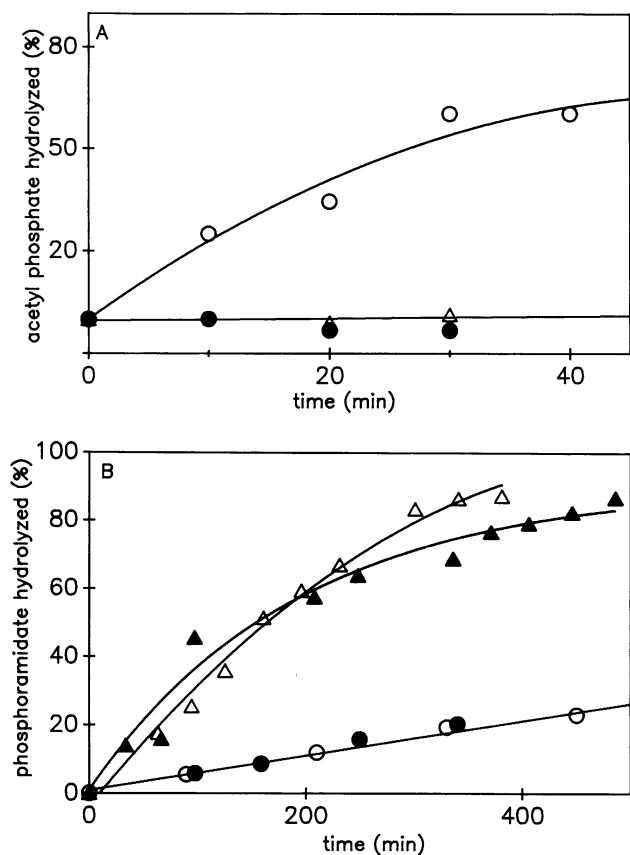


FIG. 4. (A) Acetyl phosphatase activity observed when 48  $\mu\text{M}$  CheY ( $\circ$ ), 34  $\mu\text{M}$  CheB ( $\bullet$ ), or 28  $\mu\text{M}$  CheZ ( $\Delta$ ) was incubated with 10 mM acetyl [ $^{32}\text{P}$ ]phosphate. Reactions were stopped, and the components separated by chromatography on PEI-cellulose sheets. After autoradiography, the spots corresponding to acetyl phosphate were excised for scintillation counting. (B) Phosphoramidase activity. All samples were in 100 mM Tris-HCl, pH 7.9/10%  $^2\text{H}_2\text{O}$ . Each contained 18 mM potassium phosphoramidate with the following additions: none ( $\circ$ ); 16 mM  $\text{MgCl}_2$  ( $\bullet$ ); 29  $\mu\text{M}$  CheY and 16 mM  $\text{MgCl}_2$  ( $\Delta$ ); 29  $\mu\text{M}$  CheB and 16 mM  $\text{MgCl}_2$  ( $\blacktriangle$ ). The concentration of phosphate and phosphoramidate at any given time point was determined by integration of their respective  $^{31}\text{P}$  NMR signals.

stimulation of its receptor methylesterase activity (11). Phosphoramidate, in the complete absence of the kinase, had a similar effect (Fig. 5). Of the three phospho-donors that phosphorylate CheY, only phosphoramidate activated CheB. The relative inactivity of CheB in the presence of acyl phosphates is consistent with the lack of CheB phosphorylation by acetyl [ $^{32}\text{P}$ ]phosphate (Fig. 3) and the absence of CheB acyl phosphatase activity (Fig. 4).

## DISCUSSION

We have shown that response regulators can be phosphorylated and activated in the absence of any auxiliary proteins. This demonstrates that phosphotransfer is catalyzed by the response regulators. The conserved residues and metal ion at the active site of the response regulators may now be evaluated in regard to the mechanism of phosphotransfer. A passive role of the protein-histidine kinases in phosphotransfer is consistent with the observation that a phosphorylated N-terminal fragment of CheA lacking the conserved histidine kinase domain can donate phosphate to CheY (28). The autophosphorylating activity of response regulators raises the possibility that small-molecule phospho-donors may provide a kinase-independent mechanism for regulation of adaptive responses.

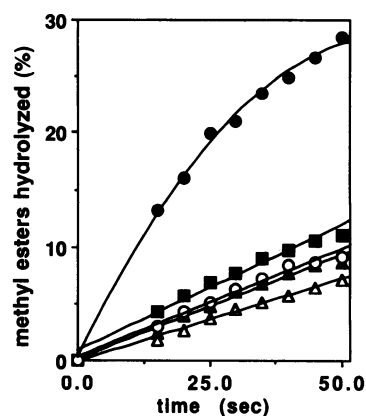


FIG. 5. Effect of potential phospho-donors on CheB receptor methylesterase activity. Esterase assays were performed with 3.1  $\mu\text{g}$  of CheB protein in 0.25 ml of 50 mM Tris-HCl, pH 7.0/5 mM  $\text{MgCl}_2$ /1 mM dithiothreitol, as described (27). *E. coli* membranes with [ $^3\text{H}$ ]methyl-labeled Tar receptors (65,000 cpm per assay) were used as substrate. Reaction mixtures were incubated at 25°C in an Eppendorf repeater pipet to facilitate rapid sampling. Time points were obtained by pipetting 20- $\mu\text{l}$  aliquots into 10  $\mu\text{l}$  of glacial acetic acid. Released [ $^3\text{H}$ ]methanol was detected by vapor-phase diffusion.  $\circ$ , No additions;  $\bullet$ , 1 mM phosphoramidate;  $\Delta$ , 1 mM acetyl phosphate;  $\blacktriangle$ , 1 mM carbamoyl phosphate;  $\blacksquare$ , 1.0 mM creatine phosphate.

Kinase-independent activation of CheY has previously been suggested based on the effects of acetate on the swimming behavior of *E. coli* (17). A motile strain that lacks all of the chemotaxis components except CheY is smooth-swimming. Addition of acetate to this strain induces tumbling behavior. This result may now be understood in terms of phosphorylation of CheY by acetyl phosphate. There are two pathways for the production of acetyl phosphate from extracellular acetate: one uses acetyl-CoA synthetase to produce acetyl-CoA, which can then be converted to acetyl phosphate by phosphate acetyltransferase; the other pathway utilizes acetate kinase to directly generate acetyl phosphate. Acetate uptake via acetyl-CoA synthetase may be favored compared with acetate uptake via the kinase since the former pathway uses two ATP equivalents while the latter only uses one. The kinase reaction may work primarily in reverse to generate ATP. Acetate-induced tumbling behavior appeared to be dependent on acetyl-CoA synthetase rather than acetate kinase (17).

Small-molecule phospho-donors may also be important in other systems. The phosphate regulon is controlled by two histidine kinases, PhoR and PhoM, and a transcriptional regulator, PhoB. Overproduction of acetate kinase can restore expression of phosphate-mediated gene expression in a strain lacking both PhoM and PhoR (29). Phosphorylation of PhoB by increased levels of acetyl phosphate could explain this observation. In the osmoregulation system, expression of the two porins, OmpF and OmpC, is under the control of a histidine kinase, EnvZ, and a transcriptional activator, OmpR. Phospho transfer between EnvZ and OmpR has been demonstrated *in vitro*. However, in an *envZ* mutant, Forst *et al.* (19) were able to detect high levels of phospho-OmpR. A possible explanation for this observation is that OmpR was phosphorylated by low molecular weight phospho-donors. In support of this explanation, preliminary experiments using acetyl [ $^{32}\text{P}$ ]phosphate as phospho-donor have demonstrated OmpR phosphorylation. In the nitrogen regulation system, transcription of the glutamine synthetase gene, *glnA*, is regulated by a histidine kinase, NtrB, and a transcriptional regulator, NtrC. It has been shown that *in vitro* transcription of *glnA* requires phospho-NtrC (38). However, elimination of the histidine kinase, NtrB, has little effect on the *in vivo* level of glutamine synthetase expression (15). Cross-talk from

other histidine kinases has been used to explain activation of response regulators in the absence of their cognate kinases. Phosphorylation of response regulators by low molecular weight phospho-donors provides an additional mechanism for this activation.

In an environment containing multiple potential phospho-donors, specificity of response regulators for particular donor pools would be necessary for appropriate physiological responses. CheY and CheB exhibit substrate specificity for small-molecule phospho-donors. CheY can use acetyl phosphate, carbamoyl phosphate, and phosphoramidate; CheB uses only phosphoramidate. Neither protein is phosphorylated in the presence of nucleoside triphosphates or creatine phosphate. Specificity is also apparent with protein phospho-donors. In *in vitro* cross-talk experiments, the rates of phosphotransfer between heterologous kinases and regulators vary dramatically (12–14).

The specificity of a given response regulator for particular phospho-donors suggests that these small molecules may provide metabolic inputs that control regulators in response to the internal state of the cell. It has been assumed that the flow of phosphate in all “two-component” systems is from the histidine kinase to the response regulators. The observation that response regulators can be phosphorylated in the presence of low molecular weight phospho-donors suggests that sometimes the flow of phosphate may proceed from small molecules to response regulators. Histidine kinases frequently function as phosphatase-activating proteins to facilitate the dephosphorylation of response regulators. In some cases, this may be their principal regulatory role. Phosphorylation of response regulators by low molecular weight phospho metabolites may help adjust long-term steady-state levels of activated response regulators.

Changes in the concentrations of phospho metabolites may also elicit short-term responses in other signal-transduction pathways. *E. coli* chemotaxis to sugars transported by the phosphoenolpyruvate:glycose phosphotransferase system (PTS) does not depend on the membrane receptor/transducers that control the CheA kinase (30). Instead these sugars are sensed by components of the PTS (31). Fluctuations in acetyl phosphate concentrations in response to changing concentrations of PTS sugars may provide an input for chemotaxis to these nutrients. It has been shown that acetate kinase can act as a phospho-donor for the PTS (32). The utilization of pyruvate to produce ATP via acetyl phosphate and acetate kinase is an important energy-generating mechanism in sugar metabolism, especially under conditions where oxygen is limiting (33).

The discovery that low molecular weight phospho-donors can be used to phosphorylate response regulators will facilitate the physical and biochemical characterization of these enzymes. Since the kinase is no longer required for phosphorylation, the activated proteins may now be studied in isolation. For instance, the results reported here show unequivocally that the autophosphatase activity of CheY and its enhancement by CheZ can occur independently of CheA and that phosphorylation *per se* is sufficient for the activation of the CheB methyltransferase.

The effect of phosphorylation on the intrinsic fluorescence of CheY is consistent with the proposal that activation of CheY involves a conformational change triggered by protein phosphorylation (4). Generation of a phosphoaspartic residue in a number of different ion-translocating ATPases induces a conformational change that provides a crucial energy-coupling step for ion transport (34, 35). An analogous conformational transition may operate in response regulators to provide a phospho-activated switch mechanism. Like CheY, the ion-translocating ATPases can use acetyl phosphate as a phospho-donor (36, 37).

In summary, we have shown that both CheY and CheB contain the necessary catalytic residues for their own phosphorylation. In addition, the generation of phosphorylated response regulators with low molecular weight phospho-donors will facilitate further characterization of these activated proteins in the absence of auxiliary kinases. Finally, the possibility has been raised that low molecular weight phospho metabolites could have a significant role in the regulation of signal-transduction pathways.

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