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Feedback Regulation of Map Kinase Signal Pathways [and Discussion]

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Feedback regulation of map kinase signal pathways

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SUMMARY

Ste7 is a MEK (MAPK/ERK kinase) family member that functions in the pheromone induced mating response pathway of *Saccharomyces cerevisiae*. We analysed the catalytic competence and *in vivo* function of Ste7 variants that have alterations of stimulatory and feedback phosphorylation sites. These analyses led us to unanticipated insights into two separate feedback mechanisms that impede the output of the mating response MAPK activation pathway.

1. INTRODUCTION

Extracellular molecules that regulate cell proliferation and differentiation in eukaryotes depend on pathways that detect signals at the cell surface and transmit them through the cytoplasm to nuclear and other intracellular targets. One mechanism for intracellular signal transmission uses a protein kinase cascade that is conserved in organisms as diverse as yeast and humans. The three sequentially acting protein kinases of the conserved cascades are members of the MEKK (MEK kinase), MEK (MAPK/ERK kinase) and MAPK (mitogen-activated protein kinase) families.

In the budding yeast, *S. cerevisiae*, four MAPK activation pathways have been identified and found to regulate different physiological processes (figure 1; for a review, see Levin & Errede 1995). One pathway controls the mating response between haploid cells of opposite mating types. Another controls a dimorphic transition to a pseudohyphal form in the diploid cell type and an invasive growth behaviour in haploid cell types. A cell integrity pathway operates to control cell wall construction, a process that is especially important at regions of polarized growth during cell proliferation or mating differentiation. A stress response pathway regulates cellular responses to high osmolarity of the extracellular medium. Distinct MAPK activation pathways also have been identified in vertebrate cells (figure 1). The most extensively studied pathway in mammalian cells controls proliferative responses to a diverse array of mitogenic agents (for a review, see Marshall 1994). Two additional pathways that control responses to stress such as exposure to ultraviolet (UV) radiation or treatment with tumor-necrosis factor α have more recently been defined (Freshney *et al.* 1994; Han *et al.* 1994; Kyriakis *et al.* 1994; Minden *et al.* 1994; Rouse *et al.* 1994; Sanchez *et al.* 1994; Yan *et al.* 1994; Derijard *et al.* 1995).

Given these separately functioning but structurally related signalling modules that coexist in the cell, there must be specialized mechanisms to insulate one cascade from spurious activation by the stimulus of another. In addition, there are likely to be common regulatory principles that control the unstimulated activity of the different cascades as well as the amplitude and duration of their induced activity. We have focused studies on the MAPK activation cascade that controls mating differentiation in budding yeast to address these issues.

2. OVERVIEW OF THE BUDDING YEAST MATING RESPONSE SIGNALLING PATHWAY

Mating occurs when the peptide pheromone secreted by one cell type binds to the specific integral membrane receptor on the opposite cell type. This cell-surface interaction stimulates dissociation of a heterotrimeric G protein into its $G_{\beta\gamma}$ and G_{α} subunits (figure 1) (see Levin & Errede (1995) and references therein). Free $G_{\beta\gamma}$ stimulates a phosphorylation cascade composed of protein kinases that function in the order Ste20, Ste11, Ste7, Fus3/Kss1. The mechanisms underlying activation of Ste20 and Ste11 (a MEKK) are still not known. However, Ste7 is directly phosphorylated and activated by Ste11 (Neiman & Herskowitz 1994). Ste7 activates Fus3 and Kss1 (MAPKs) by phosphorylating them at the Thr and Tyr residues of the signature TEY motif within the MAPK phosphorylation lip (Errede *et al.* 1993). Fus3 and Kss1 are redundant for pheromone induced signal transmission leading to transcriptional activation of genes whose products are required for the process of mating (Elion *et al.* 1991). Fus3 has an additional role in promoting arrest of the cell cycle at the G_1 phase (Peter *et al.* 1993; Tyers & Futcher 1993).

The mating response pathway uses a protein called Ste5, whose function has been enigmatic until recently. Genetic analyses showed that Ste5 function was first required after the G protein but before or at the level of Ste11 (for review, see Levin & Errede 1995). However, two-hybrid interaction studies showed that Ste5 interacts with Ste11, Ste7, Kss1 and Fus3 (Choi *et al.* 1994; Marcus *et al.* 1994; Printen & Sprague 1994). The multiple interactions are consistent with Ste5 having a role in several steps along the MAPK activation cascade. Additional biochemical evidence

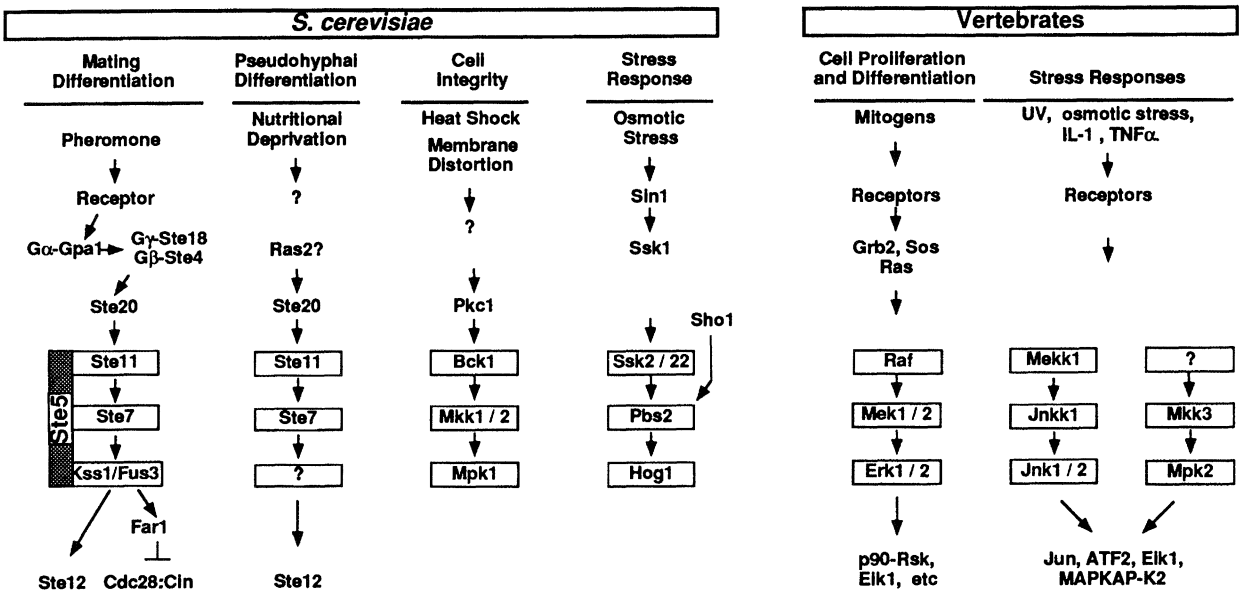

Figure 1. MAKP-dependent singal transduction pathways in *S. cerevisiae* and vertebrates. Homologous kinases of the conserved activation modules are indicated within boxes at equivalent tiers in the pathways.

showed that Ste5 and the four kinases are present in a macromolecular assembly (Choi *et al.* 1994). These findings suggest a model in which Ste5 is a scaffold for the kinases of the activation module. This physical organization could facilitate both stimulatory and down-regulatory interactions between the kinases. It may also minimize cross-interactions between the kinases of the mating response pathway and those of other MAPK modules. Support for the latter role has come from the behaviour of a gain-of-function Ste7 variant, whose ability to function within other pathways (such as the cell integrity pathway) is restricted by the presence of Ste5 (Yashar *et al.* 1995).

The responsiveness of a signal transduction system to a persistent stimulus diminishes with time. This phenomenon, called desensitization or adaptation, is a critical aspect of a coherent response to a given stimulus. For example, the pheromone induced signal is rapidly turned off after a zygote is formed by the fusion of two mating partners or when cells are exposed to pheromone but do not find a mating partner. In both circumstances, cells reenter the cell-cycle and resume proliferation. Several different molecular mechanisms for adaptation have already been described. These inclue inactivation of the pheromone by a specific protease, endocytosis and degradation of the α-pheromone receptor complex and regulatory events that antagoize singal transmission functions at the level of the MAPKs (Ciejek & Thorner 1979; Chan & Ott 1982; Jenness & Spatrick 1986; Reneke *et al.* 1988; Doi *et al.* 1993). The experiments we describe point to a role of feedback phosphorylation in the attenuation of the mating response pathway signal.

3. STE7 PHOSPHORYLATION STATES AND CATALYTIC ACTIVITY

Previous studies on Ste7 showed that pheromone stimulation induces its enzyme activity and its hyperphosphorylation (figure 2, lanes 1–4 upper and lower panels, respectively) (Errede *et al.* 1993; Zhou *et al.* 1993). Ste7 hyperphosphorylation, which is readily monitored on immune blots by a mobility shift of the protein, depends on pheromone induction and the

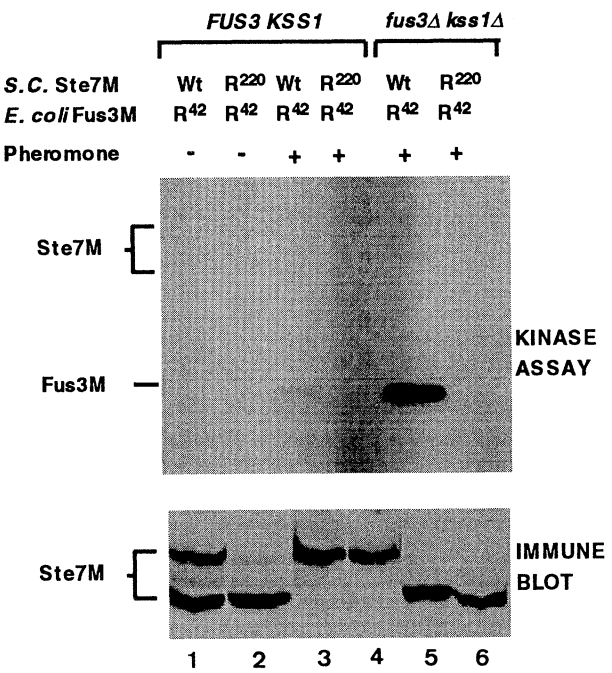

Figure 2. Ste7 activity and phosphorylation states. Upper panel: immune complex kinase assays comparing the amount of Fus3-R^{42} phosphorylation catalysed by Ste7 (Wt) and kinase-inactive Ste7-R^{220} (R^{220}). These proteins were expressed in *FUS3 KSS1* strain K2180 and *fus3*Δ*kss1*Δ strain K2314 (Errede *et al.* 1993) as indicated. Cultures were induced by mating pheromone (+) or not induced (−) prior to extract preparation. Lower panel: immune blot of Ste7 or Ste7-R^{220} extracts used in the phosphorylation assays above. The fast mobility (lower) band corresponds to unphosphorylated or underphosphorylated Ste7. The slow mobility (upper) band is hyperphosphorylated Ste7. Extract preparation, immune affinity purification, phosphorylation assays and immune detection were as described (Errede *et. al.* 1993; Zhou *et al.* 1993).

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presence of the down stream MAPKs, Fus3 and Kss1 (figure 2, bottom panel). Ste7 isolated from strains that lack Fus3 and Kss1 (*fus3*Δ *kss1*Δ) was not hyperphosphorylated and exhibited higher activity than when isolated from strains expressing the downstream MAPKs (*FUS3 KSS1*) (figure 2, lanes 3–5). These results show that Ste7 hyperphosphorylation is not a prerequisite for its activation but is instead a consequence of it. A further implication is that the Fus3/Kss1-dependent hyperphosphorylation of Ste7, directly or indirectly, attenuates its activity.

Ample evidence supports the conclusion that phosphorylated residues different from those causing the mobility shift on SDS-polyacrylamide gel electrophoresis are nevertheless essential for Ste7 catalytic competence. For example, the activity of Ste7 isolated from pheromone induced *fus3*Δ *kss1*Δ strains was abolished by treatment with the serine/threonine protein phosphatase 2A (A. Gartner & B. Errede, unpublished). Also, biochemical reconstitution experiments verified that Ste11 directly phosphorylates Ste7 and that this modification is sufficient for its activation (Neiman & Herskowitz 1994; B. Errede, unpublished).

Taken together these studies suggest that there are two types of Ste7 regulatory phosphorylations. One class is catalysed by Ste11 and is required for catalytic competence. The other class is catalysed in a feedback reaction by downstream kinase(s) and is associated with attenuation of Ste7 activity.

4. STIMULATORY PHOSPHORYLATIONS OF STE7

To further explore regulatory inputs that might influence transitions between the different regulatory states of Ste7, we analysed the *in vivo* signalling characteristics of Ste7 variants with alterations in the presumed regulatory phosphorylation sites. Identification of the activating phosphorylation sites in other Meks made it likely that Ste11 would phosphorylate the analogous sites in Ste7 (serine 359 and threonine 363) (Alessi *et al.* 1994; Cowley *et al.* 1994; Zheng & Guan 1994). We made alanine substitutions at both residues and expressed the variants in a tester strain that has a chromosomal deletion of *STE7* (*ste7*Δ) and the *FUS1::HIS3* reporter gene. The amount of reporter gene product, His3, was used as an indicator of the transcriptional response supported by different variants in the tester strain. Assessment of His3 production was by a simple growth assay on medium lacking exogenous histidine. As expected, the Ste7-A^{359} and Ste7-A^{363} variants did not support expression of the *FUS1::HIS3* reporter gene (table 1). Mating and G_1 arrest assays gave the same results (Zhou 1993; Q.-y. Ge, unpublished).

In the case of Mek1, glutamate substitutions at the phosphorylation site residues rendered the variants catalytically competent in the absence of modification by Raf (Cowley *et al.* 1994; Mansour *et al.* 1994; Seger *et al.* 1994). To make the analogous test with Ste7, we compared the catalytic activity of purified Ste7 with that of glutamate substitution variants using kinase-inactive Fus3-R^{42} as substrate. We chose to use only proteins expressed and purified from *E. coli* so that there would be no possibility of activation by contaminating yeast proteins. Because the full length Ste7 is not stable when expressed in *E. coli*, this approach required us to use derivatives of Ste7ΔS, a deletion variant which is nevertheless catalytically active when isolated from yeast (Errede *et al.* 1993). Using *E. coli* expressed enzymes, there was no phosphorylation of Fus3-R^{42} above background in reactions with the negative control (Ste7ΔS-R^{220}), Ste7ΔS, or either single glutamate substitution variant (Ste7ΔS-E^{359}, Ste7ΔS-E^{363}) (figure 3, lanes 1–5). The double glutamate substitution derivative (Ste7ΔS-$E^{359}E^{363}$) was the only Ste7ΔS variant that was catalytically competent (figure 3, lane 6). This outcome suggests that modi-

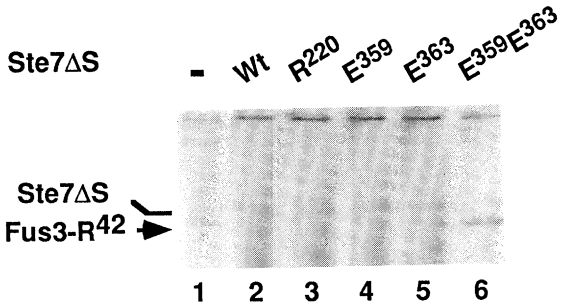

Figure 3. Comparison Ste7ΔS and glutamate substitution derivatives in phosphorylation assays. Kinase-inactive Fus3-R^{42} was used as substrate for reactions without (−) or with Ste7ΔS (Wt) or derivatives with amino acid substitutions as indicated. All proteins were expressed and purified from *E. coli* as GST fusions as described; after purification, the GST polypeptide was removed from the fusion by treatment with thrombin (see Errede *et al.* (1993) for methods).

Table 1. *Transcriptional response supported Ste7 and variants in different genetic backgrounds*

	His3 Expression[a]				
host strain[b] variant[c]	*ste7*Δ	*ste7*Δ *ste11*Δ	*STE7* *ste4*Δ	*STE7* *ste5*Δ	STE7 *ste11*Δ
Ste7	+	−	−	−	−
Ste7-A^{359}	−	−	−	−	−
Ste7-A^{363}	−	−	−	−	−
Ste7-$E^{359}E^{363}$	−	++	++	++	++
Ste7-A^{471}	+	−	−	−	−
Ste7-$E^{359}E^{363}A^{471}$	−	++	++	++	++
Ste7ΔS	+	−	−	−	−
Ste7ΔS-A^{471}	+	−	−	−	−
Ste7ΔS-$E^{359}E^{363}$	+	+++	++	+++	++
Ste7ΔS-$E^{359}E^{363}A^{471}$	+	+++	++	+++	++

[a] Relative amounts of His3 are indicated by the highest concentration of amino triazole (AT) tested that allowed growth on -His plates. (+, no AT; ++, 5 mM AT; +++ 20 mM AT.)

[b] All strains are isogenic to SY2003 (*MATα HIS3::FUS1::HIS3 mfa2-Δ1::FUS1-lacZ ade1 leu2–3,112 trp1-DH1 ura3–52*) except for the indicated deletion alleles (Yashar *et al.* 1995).

[c] The centromeric plasmid pNC318 or derivatives of pNC318 with nucleotide substitutions corresponding to indicated amino acid replacements were used for expressing Ste7 or its variants (Zhou *et al.* 1993).

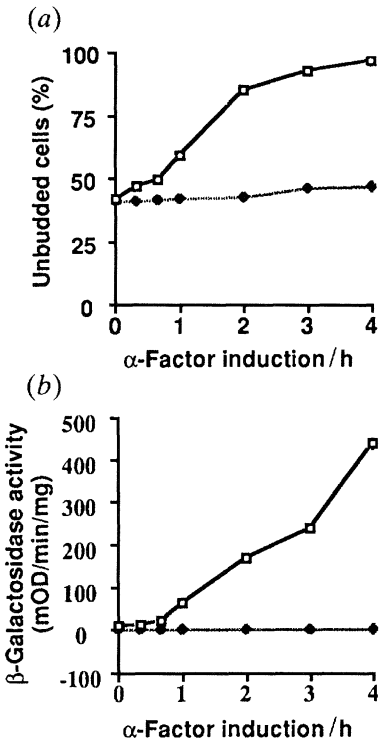

Figure 4. Comparison of pheromone induced responses in strains expressing Ste7 or Ste7-$E^{359}E^{363}$. Cultures of exponentially growing E929–6C-18 (*ste7*Δ *FUS1-lacZ*) with centromeric plasmids expressing Ste7 (□) or Ste7-$E^{359}E^{363}$ (◆) were treated with α-factor (5 μM). Aliquots were removed at appropriate intervals. (*a*) Pheromone-induced G_1 arrest measured by the percentage of unbudded cells. (*b*) Pheromone-induced expression of the *FUS1-lacZ* reporter gene measured by the activity of its product, β-galactosidase, in extracts prepared from samples.

fication of both serine 359 and threonine 363 is required for Ste7 catalytic competence. The result is also consistent with the observed loss of function phenotype associated with alanine substitution at either serine 359 or threonine 363 (table 1).

Although the mutatational analyses identified Ste7 regulatory phosphorylation sites as anticipated from analogies with other MEK family members, the outcome is different that predicted by reconstitution experiments. Only threonine 363 of Ste7 was significantly modified by Ste11 *in vitro* (Neiman & Herskowitz 1994). The failure to detect Ser 359 phosphorylation could be a consequence of the poor activity of Ste11 *in vitro*. Alternatively, the alanine 359 substitution might be adversely affecting Ste7 activity and the glutamate 359 substitution promoting activity for reasons independent of phosphorylation by Ste11.

We anticipated that the constitutive Ste7-$E^{359}E^{363}$ enzyme would activate mating pathway responses independently of an inductive signal. To our surprise, Ste7-$E^{359}E^{363}$ was non functional with respect to G_1 arrest and transcriptional responses when expressed in cells with an otherwise wild-type signal pathway (figure 4*a*, *b*; table 1). Paradoxically, the Ste7-$E^{359}E^{363}$ variant did activate a transcriptional response independently of an inductive signal in strains with deletions of genes encoding signalling components that act at the same level or upstream of Ste7 (table 1, *ste5*Δ, *ste11*Δ, *ste4*Δ). These results reveal that there are mechanism(s) by which upstream components of the mating response pathway inhibit constitutive signalling by Ste7.

5. FEEDBACK HYPERPHOSPHORYLATION OF STE7

We reasoned that hyperphosphorylation might contribute to the inhibition of signalling observed when Ste7 activity is consitutive. To apply a mutational strategy to this possibility, it would be helpful to know if Fus3/Kss1 directly phosphorylate Ste7 or if other kinases need to be considered. Toward this end, a Ste7 N-terminal polypeptide (Ste7-N) and a catalytic domain polypeptide (Ste7ΔS) were used as potential substrates for Fus3 in phosphorylation assays (figure 5*a*). Each reaction mixture contained autoactivated Fus3, [^{32}P]-ATP and Ste7-N or Ste7ΔS polypeptides as substrate. Both the Ste7-N and Ste7ΔS polypeptides were phosphorylated by Fus3 but not by the catalytically incompetent variant (Fus3-R^{42}) (figure 5*b*). These results are consistent with the notion that Ste7 hyperphosphorylation is directly catalysed by the downstream MAPKs.

Results from phosphopeptide and phosphoamino acid mapping experiments implicate at least three serine and three threonine residues within the Ste7-N polypeptide as Fus3 phosphorylation sites. The different mobility species of Ste7-N seen after Fus3 phosphorylation correspond to modification of different subsets of these sites (figure 5*b*, lane 4). By contrast Fus3 phosphorylates Ste7ΔS predominantly at a single residue, serine 471 (figure 5*b*, lanes 5 and 6). The predominance of phosphorylation in the N-terminal region is consistent with results from *in vivo* labelling studies showing greatly decreased amounts of pheromone induced phosphorylation of Ste7ΔS compared with Ste7 (B. Errede, unpublished).

The simple model that hyperphosphorylation attenuates Ste7 activity leads to the prediction that deletion or alanine substitutions at the sites modified by Fus3 might result in a variant with enhanced activity compared with the wild-type enzyme. To test this prediction, we expressed Ste7-A^{471}, Ste7ΔS, and Ste7ΔS-A^{471} variants from a centromeric plasmid in the tester strain that has a chromosomal deletion of *STE7* (*ste7*Δ) and the *FUS1::HIS3* reporter gene. No significant difference between Ste7 or any of the variants was revealed by the growth assay for assessment of *FUS1-HIS3* expression (table 1). In addition, there was no difference between Ste7ΔS and Ste7 in quantitative measurements of *FUS1::lacZ* reporter gene expression or G_1 arrest during pheromone induction (Zhou 1993). These results do not support the simple prediction that the hyperphosphorylation region and/or serine 471 attenuate Ste7 activity during a normal pheromone response.

We then asked if the feedback phosphorylation regions might be involved in the inhibition of Ste7-$E^{359}E^{363}$ activity in strains with an intact signalling pathway. To make these tests, we generated variants that combined the glutamate substitutions at positions 359 and 363 with alterations that preclude feedback

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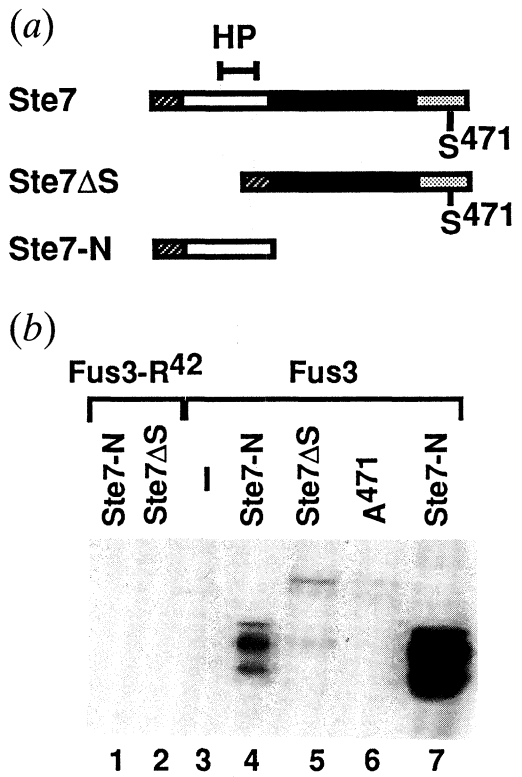

Figure 5. Reconsitution of Fus3 feedback phosphorylation of Ste7. (*a*) Diagrams showing the N-terminal (▦), the S (□), catalytic (■), and C-terminal domains (▨) of Ste7. The hyperphosphorylation (HP) region within the S domain is indicated by the bar above the diagram. (*b*) Phosphorylation assays showing Fus3 phosphorylation of Ste7-N or Ste7ΔS polypeptides. All proteins were expressed and purified from *E. coli* as a GST fusions. Subsequent to isolation the GST polypeptide was removed from each fusion by thrombin treatment. The *E. coli* purified Fus3 was autoactivated incubation in cold ATP for 2 h prior to addition of ^{32}PATP and the Ste7-N or Ste7-ΔS polypeptide as substrate. Lane 4 has one-tenth the sample of lanes 1–3 and 5–7.

phosphorylation by Fus3. The signalling competence of different derivatives was compared using the *FUS1::HIS3* reporter gene assays as before.

In contrast to Ste7-E^{359}E^{363}, the Ste7ΔS-E^{359}E^{363}derivative was signalling competent in the *ste7*Δ strain with an otherwise intact mating response pathway (table 1). These circumstances reveal that the N-terminal hyperphosphorylation domain has a negative influence on Ste7-E^{359}E^{363} activity. Because the alanine substitution at position 471 made no difference in the activity of the full length or N-terminal deletion derivative (Ste7-E^{359}E^{363}A^{471} or Ste7ΔS-E^{359}E^{363}A^{471}), serine 471 does not appear to contribute to the attenuation of Ste7 activity (table 1). Interestingly, signal activity of Ste7ΔS-E^{359}E^{363} was also higher than Ste7-E^{359}E^{363} in strains with deletions of upstream signal pathway components (*ste11*Δ, *ste5*Δ and *ste4*Δ) (table 1). Therefore, the hyperphosphorylation domain acts independently of the upstream components.

6. CONCLUSIONS AND PERSPECTIVES

These studies reveal that the N-terminal hyperphosphorylation domain negatively influences Ste7 function under the unusual circumstance where signal output is uncoupled from pheromone stimulation. However, the hyperphosphorylation domain has a negative effect on Ste7 function whether the upstream pathway is intact or disrupted. This additional result suggests that there are at least two independent mechanisms for down regulating constitutive Ste7 activity. One is dependent on the hyperphosphorylation domain of Ste7 and the other on upstream components of the mating response pathway. The latter mechanism may also involve feedback phosphorylation because upstream components such as Ste5 are phosphorylated by Fus3/Kss1 (Kranz *et al.* 1994). Our current understanding of the mating response pathway is that Ste5 serves as a scaffold to organize the kinases of the mating pathway and interacts with the G_β subunit. Given this model, it is tempting to speculate that feedback phosphorylation of multiple components alters their association and impedes signal transmission.

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Discussion

Question. Are there specific STE5-like proteins for the different kinase pathways?

B. Errede. At present, STE5 is the only properly identified protein of this type. I have heard rumours that there is a SH3-containing protein that might perform a similar function in the stress response pathway, and our laboratory has indirect indications that there might be an STE5-like protein that functions in the pseudohyphal pathway in diploid cells. In this case, STE7 and STE11 might be able to interact with alternative scaffold proteins of the STE5 type in order to participate in the two pathways.

C. J. Marshall (*Chester Beatty Laboratory, Institute of Cancer Research, London, U.K.*). Can you reconstitute the STE5 function *in vitro* with isolated kinases and the STE5 protein?

B. Errede.We are trying to put together the reagents for such an experiment, but no-one has yet achieved this result.

M. Karin (*Department of Pharmacology, University of California, San Diego, U.S.A.*). Do any of the pathways operate differently in haploid and diploid cells?

B. Errede. The cell integrity pathway and the stress response pathway operate identically in haploid and diploid cells. The pseudohyphal response operates only in diploid cells, and mating occurs only in haploids. There is a related response to nutrient deprivation called invasive growth in haploid cells which might involve a modified version of the pseudohyphal pathway.

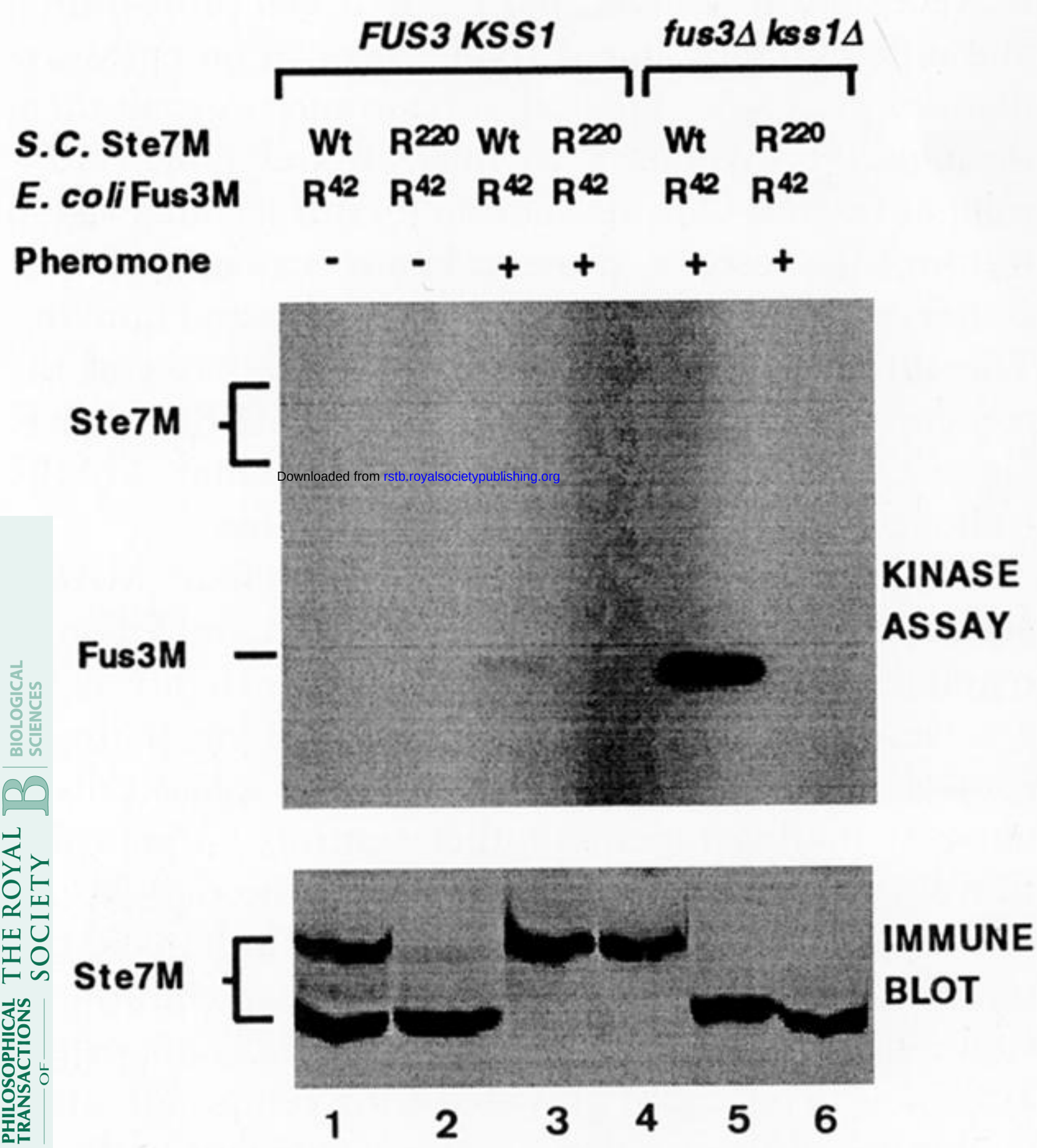
P. J. Parker (*Imperial Cancer Research Fund, London, U.K.*). Does STE5 have any role in intracellular localization of the components of the kinase pathway, and is there turnover of the kinase components on STE5 during activation? For example, does the final component of the pathway rapidly cycle on and off STE5, and if so what is the speed of this cycling?

B. Errede. The work on localization has not progressed very far. However, there is said to be evidence coming from Jeremy Thorner's laboratory indicating that STE5 gets localized in the nucleus, that the KSS1 MAPK also gets nuclear-localized, and that FUS3 is more diffusely distributed but becomes more focused towards the nucleus after pheromone stimulation. We have a preliminary indication that STE7 concentrates in the region of the nucleus during pheromone stimulation.

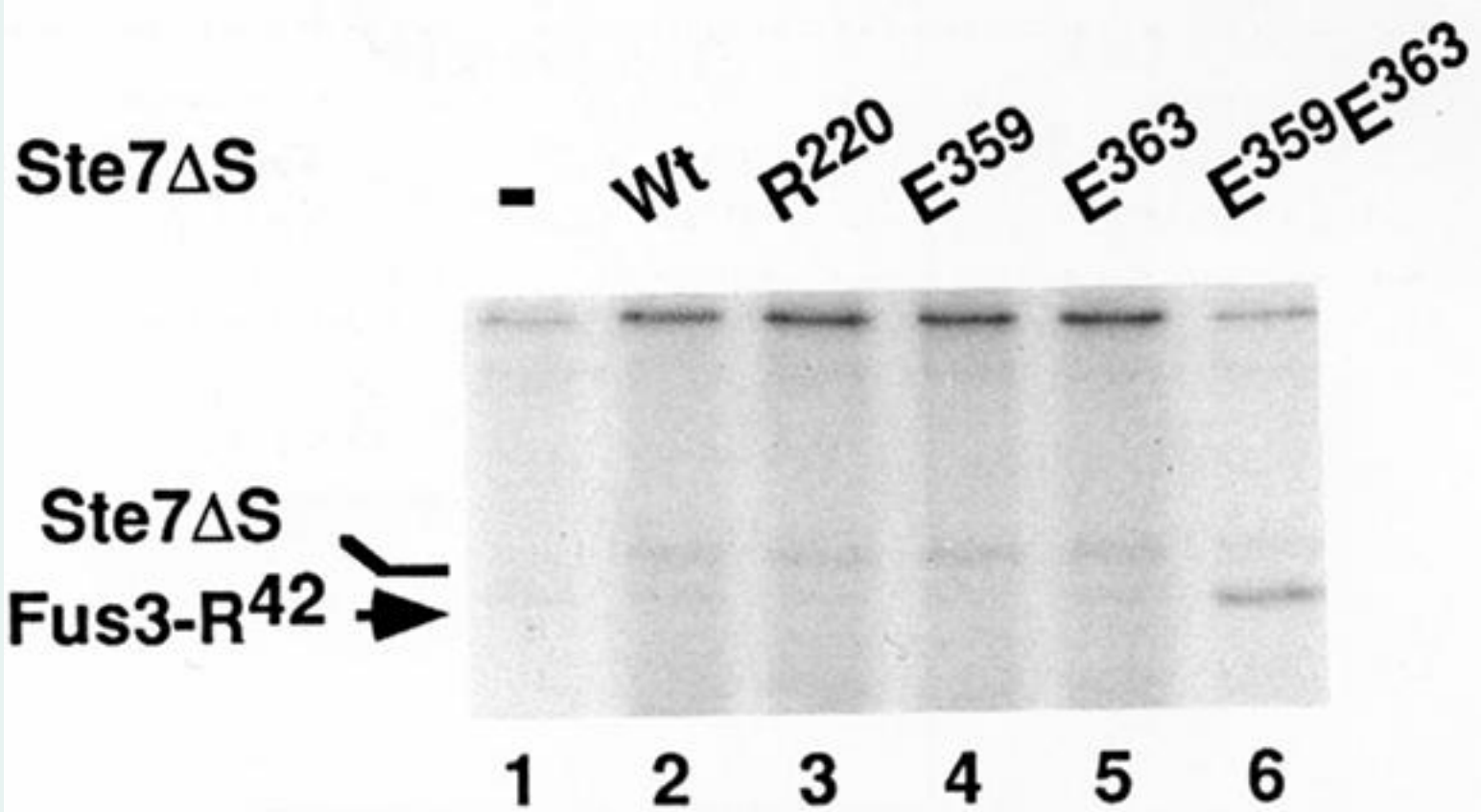
When one looks at the stoichiometry of the various components of the pathway, the FUS3/KSS1 MAPKs are 100–200-fold more abundant than the upstream kinases. I therefore think that the complex is probably acting as 'an enzyme', with the MAPK component cycling on and off a complex of STE5, STE7 and

STE11. If this were not the case, there would be no obvious reason why the whole pathway could not be incorporated into a single multi-enzyme complex. Maybe the organization exists to give multiple sites of regulation, acting as 'a rheostat' to fine-tune the amplitude and duration of the output signal.

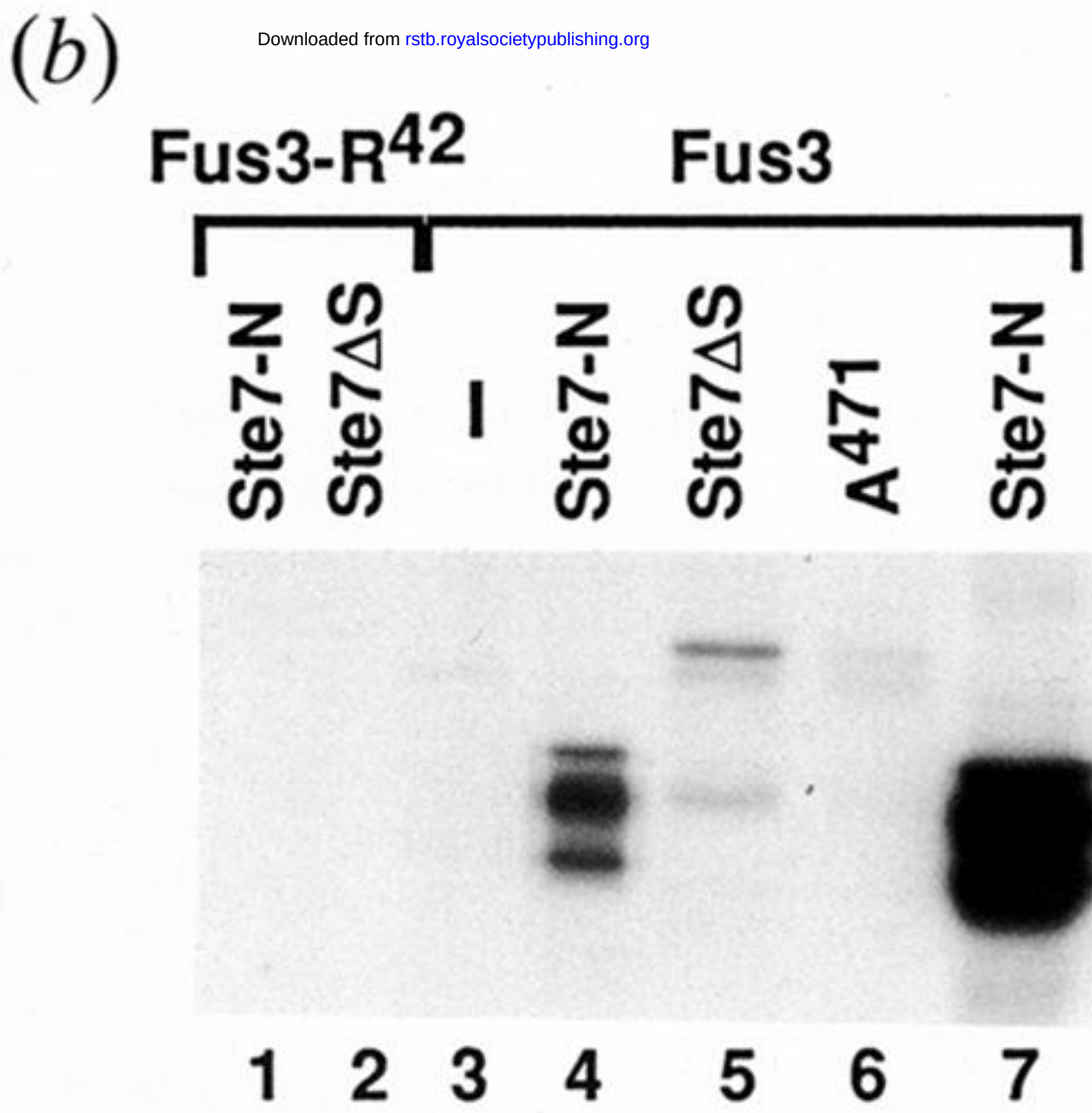
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



gure 2. Ste7 activity and phosphorylation states. Upper nel: immune complex kinase assays comparing the amount Fus3-R^{42} phosphorylation catalysed by Ste7 (Wt) and nase-inactive Ste7-R^{220} (R^{220}). These proteins were expres-l in *FUS3 KSS1* strain K2180 and *fus3Δkss1Δ* strain K2314 rrede *et al.* 1993) as indicated. Cultures were induced by ating pheromone (+) or not induced (−) prior to extract eparation. Lower panel: immune blot of Ste7 or Ste7-R^{220} tracts used in the phosphorylation assays above. The fast obility (lower) band corresponds to unphosphorylated or derphosphorylated Ste7. The slow mobility (upper) band hyperphosphorylated Ste7. Extract preparation, immune inity purification, phosphorylation assays and immune tection were as described (Errede *et. al.* 1993; Zhou *et al.* 93).



gure 3. Comparison Ste7ΔS and glutamate substitution rivatives in phosphorylation assays. Kinase-inactive Fus3-^{2} was used as substrate for reactions without (−) or with e7ΔS (Wt) or derivatives with amino acid substitutions as dicated. All proteins were expressed and purified from *E. i* as GST fusions as described; after purification, the GST lypeptide was removed from the fusion by treatment with combin (see Errede *et al.* (1993) for methods).



gure 5. Reconsitution of Fus3 feedback phosphorylation of e7. (*a*) Diagrams showing the N-terminal (▦), the S (□), talytic (■), and C-terminal domains (▣) of Ste7. The perphosphorylation (HP) region within the S domain is dicated by the bar above the diagram. (*b*) Phosphorylation says showing Fus3 phosphorylation of Ste7-N or Ste7ΔS lypeptides. All proteins were expressed and purified from *coli* as a GST fusions. Subsequent to isolation the GST lypeptide was removed from each fusion by thrombin eatment. The *E. coli* purified Fus3 was autoactivated cubation in cold ATP for 2 h prior to addition of ^{32}PATP d the Ste7-N or Ste7-ΔS polypeptide as substrate. Lane 4 s one-tenth the sample of lanes 1–3 and 5–7.