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Saccharomyces cerevisiae Ste5 Is Important for Induction and Substrate Specificity of Fus3 MAP Kinase in the Pheromone Signaling Pathway

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The pheromone pathway is one of the mitogen activated protein kinase (MAPK) signaling pathways identified in *Saccharomyces cerevisiae* and is involved in both G1 cell cycle arrest and mating of cells. Fus3 functions at a branching point for G1 cell cycle arrest and mating responses in the signaling cascade, and the Fus3 MAPK uses components of both G1 arrest and mating routes as substrates. The Ste5 is a scaffold protein of the MAPK module and is essential for the activation of Fus3. However, it is not known how Ste5 is involved in the specific activation of Fus3 in G1 arrest and mating. In this study, we characterized several G1 arrest defective Ste5 mutants to better understand the roles of Ste5 in the regulation of Fus3. The level of Fus3 increased by treatment with α-factor. However, the α-factor effects were not readily apparent in the observation of yeast cells containing G1 arrest defective *ste5* mutant. This suggests that Ste5 plays an essential role in Fus3 induction. Fus3 immune kinase assay of G1 arrest defective *ste5* transformants revealed that Ste5 is important for substrate specificity of Fus3 for G1 arrest and/or mating.

Keywords: Fus3; MAP Kinase; *Saccharomyces cerevisiae*; Signal Transduction; Ste5.

Introduction

The *Saccharomyces cerevisiae* pheromone pathway is involved in the G1 phase of cell cycle arrest and mating of cells. It is modeled because it is more completely understood than any other organism (Herskowitz, 1995). Therefore, the pheromone pathway has been used as a model system to understand the mechanism of MAPK signal transduction. Binding a mating pheromone to its receptor activates the associated G protein, thereby releasing βγ subunits from the α subunit (reviewed in Sprague Jr. and Thorner, 1993). The activated βγ subunits transmit their signal to downstream kinases including Ste20, Ste11, Ste7 and Fus3 by a series of phosphorylation reactions (Cairn *et al.*, 1992; Leeuw *et al.*, 1998; Neiman and Herskowitz, 1994; Wu *et al.*, 1995; Zhou *et al.*, 1993) and this results in two different physiological responses, G1 arrest and mating (Levin and Errede, 1995; Neiman and Herskowitz, 1994; Rhodes *et al.*, 1990). The Ste20 links G-protein-coupled receptor through Gβγ to a MAPK module (Leeuw *et al.*, 1995; Wu *et al.*, 1995), and it interacts with Ste11 and phosphorylates it *in vitro* (Wu *et al.*, 1995). The Fus3 MAPK is a dividing point for two different physiological responses in the pheromone pathway, G1 cell cycle arrest and mating (reviewed in Herskowitz, 1995). Fus3 phosphorylated the downstream target transcription factor, Ste12, for mating as well as a negative regulator of G1-cyclin/Cdc28, Far1, for G1 cell cycle arrest (Kranz *et al.*, 1994; Peter *et al.*, 1993). In addition, Fus3 also phosphorylated the upstream signaling components, Ste11 and Ste7, as well as a scaffold protein, Ste5 (Choi *et al.*, 1994; Kranz *et al.*, 1994). The roles for phosphorylation of the upstream components by Fus3 need

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Abbreviations: G protein, guanine nucleotide binding protein; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; SC, synthetic complete medium.

to be determined. Another MAPK, Kss1 shares Ste12 as the substrate with Fus3, but not Far1 (Elion *et al.*, 1991). However, recent studies have identified Kss1 as a MAPK for the filamentation-invasion pathway rather than as a MAPK for the pheromone pathway in *S. cerevisiae*. In addition, Kss1 can replace the mating function of Fus3 only in the absence of Fus3 (Cook *et al.*, 1997; Madhani *et al.*, 1997).

Currently, the mechanism of the pheromone signaling pathway has been identified as a much more complex mechanism involving a scaffold protein, Ste5, rather than as a simple linear signaling cascade (Reviewed in Gustin *et al.*, 1998; Herskowitz, 1995). Ste5 recruits many of the components of the signaling pathway including the βγ-subunit of G protein (Leeuw *et al.*, 1998), Ste20, Ste11, Ste7 as well as the MAPK, Fus3 (Choi *et al.*, 1994; 1999a; Marcus *et al.*, 1994), and it also forms a high molecular weight complex with other pheromone signaling components (Choi *et al.*, 1994; 1999a). The high molecular weight complex disappeared when Ste5 was not present, suggesting the essential requirement of Ste5 in the formation of the complex (Choi *et al.*, 1999a). A small fraction of Fus3 present in the high molecular weight complex portion was most active compared to the large portions of Fus3 present in low molecular weight fractions in glycerol gradient fractionation (Choi *et al.*, 1999a). These results suggested that the formation of the high molecular weight complex mediated by Ste5 may be required for the activation of Fus3 MAPK, and that Ste5 may affect G1 arrest and mating by the modulation of Fus3 MAPK activity. Therefore, levels of Ste5 may be important in the regulation of pheromone signal transduction. Recently, we identified Ste11 as important for protein stability of Ste5 (Kim *et al.*, 1998). Although Ste5 is involved in both G1 arrest and mating processes of cells, it is not clear whether Ste5 regulates both processes simultaneously or not. Furthermore, the roles of Ste5 in both processes and the relationship of Ste5 with Fus3 MAPK are not clear. Recently, we suggested a separate role for Ste5 in G1 arrest and mating by isolating several Ste5 mutants that lost G1 arrest function without significant loss of mating function (Choi *et al.*, 1999b). However, it is not known how Ste5 is involved in the differential regulation of the G1 arrest and mating processes.

In this study, we further present the role of Ste5 by characterizing G1 cell cycle arrest defective *ste5M* mutants. Biochemical characterization of G1 arrest defective *ste5* mutants suggested that the Ste5 protein might play important roles in the induction and discrimination of physiological substrates of Fus3 MAPK for the accurate regulation of G1 arrest and mating responses.

Materials and Methods

Media and microbiological techniques Yeast media including synthetic complete (SC) selective medium were prepared as previously described (Sherman *et al.*, 1986). YPD (2% yeast extract, 4% Bacto peptone, 2% glucose and 0.03% L-tryptophan) and YNB (0.15% yeast nitrogen base supplemented with glucose, ammonium sulfate, and inositol to 2%, 0.5%, and 0.2 mM, respectively) were also prepared as previously described (Choi *et al.*, 1999b). Standard bacterial procedures were used for bacterial transformation, plasmid DNA preparation, and plasmid construction (Sambrook *et al.*, 1989). *Escherichia coli* cells were grown on LB medium containing 50 μg/ml ampicillin for plasmid selection. Plates for each medium were prepared by adding Bactoagar to 2%. Yeast transformations were performed by Lithium acetate procedure (Ito *et al.*, 1983).

Plasmids and yeast strains The pKC29 (*FUS3 LEU2 2μ*) was constructed by inserting the *Hin*dIII fragment of *FUS3* from pYEE114 (Elion *et al.*, 1993) into the *Hin*dIII site of Yep13 (Mackay, 1983). pXT1 (*KSS1 LEU2 2μ*), pSTE7.4 (*STE7 LEU2 2μ*) and pSTE11.1 (*STE11 LEU2 2μ*) were described in previous studies, respectively (Chaleff and Tatchell 1985; Courchesne *et al.*, 1989; Teague *et al.*, 1986). The pYEE1102, a plasmid containing hemaglutinin epitope tagged *FUS3* (*FUS3-HA HIS3 CEN*), was described in a previous study (Elion *et al.*, 1993). The pKC20 (*STE5M URA3 2μ*), pKC20M69 ($ste5^{\Delta\text{-}776}$ *URA3 2μ*) or pKC20M72 ($ste5M^{D248V}$ *URA3 2μ*), and pKC20M105 ($ste5M^{Y421D}$ *URA3 2μ*) were described in a previous study (Choi *et al.*, 1999b). The EKY150 (*ste5Δ::TRP1*), EKY431 (*ste5Δ::TRP1 kss1::HIS3*), and L1543 (MAT*a lys9*) yeast cells were also described in a previous study (Choi *et al.*, 1999b). EY1093 (*ste5Δ::TRP1 fus3-8::ADE2*) was obtained from Dr. Elaine Elion at Harvard Medical School (Elion *et al.*, 1993).

Pheromone sensitivity assay and mating Standard halo assay was performed on a SC selective plate as previously described (Sprague, 1991) with some modifications. Overnight grown yeast cells were adjusted to equal numbers by dilution of the culture. One hundred ml of the diluted cells were placed into a sterile tube and 2 μl of 50°C top agar in the SC selective medium were added, then the cell-agar mixture was spread onto a selective plate (diameter = 5.5 cm). In the middle of the plate, a sterile filter disk was located and the disk was spotted with 5 μl of 167 μM α-factor dissolved in dimethyl sulfoxide. The plates were then incubated in a 30°C incubator and photographed after 1 day.

Mating assay Qualitative mating assay was performed as described previously (Choi *et al.*, 1999b); Strains to be tested were patched on SC selective plates and the cells were grown overnight in a 30°C incubator. The lawn of α mating partner cell (L1543) was also prepared by overnight growth on a YPD plate. Overnight-grown patched cells were replica-plated onto the fresh SC selective plate. L1543 mating partner cell lawn was also transferred onto the plate replicated with a tester strain. After incubation at 30°C for one day, diploid cells were selected by replica-plating the cells onto the YNB plate (Sherman *et al.*, 1986). Cell growth was photographed after one day of cell growth in a 30°C incubator.

Cell culture and preparation of yeast extracts Yeast strains containing plasmids were grown at 30°C in SC media containing 2% dextrose. For α-factor induction, cells were treated with α-factor to 50 nM at the A_{600} value of 0.6–1.0, and the cells were further grown for 1 h. Cells were collected by centrifugation and washed once with sterile distilled water. Whole cell extracts were prepared (Elion *et al.*, 1993) using modified H buffer (25 mM Tris-Cl, pH 7.4, 15 mM EGTA, 1 mM dithiothrietol, 0.1% Triton-X-100, 0.25 mM meta-vanadate, 0.25 mM ortho-vanadate, 0.1 μg/ml PMSF, and 5 μg/ml each of pepstatin A, chymostatin, leupeptin, peptin, respectively) containing 10% glycerol and 250 mM NaCl.

Western blotting Samples were electrophoresced by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels (acrylamide:bis-acrylamide ratio 30:0.8). Western blotting was performed as described (Kim *et al.*, 1998) using chemiluminescent detection kits (Genepia Inc., Korea). The 9E10 and 12CA5 mouse monoclonal antibodies for detection of Myc and HA epitopes were purchased from Santa Cruz Biotech Co. (Santa Cruz, CA) and Medical and Biological Laboratories Co., LTD (Japan), respectively. Horse radish peroxidase (HRP) conjugated anti-rabbit and anti-mouse secondary antibodies, and anti-rabbit polyclonal Fus3 antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The α-tubline antibody was also purchased from Santa Cruz Biotech Co.

Preparation of immune complex and kinase assay Typically, immunoprecipitation was performed with 5 μg of 9E10 anti-Myc or 12CA5 anti-HA monoclonal antibody. Four hundred μg of whole cell extract was diluted into modified H buffer to the final volume of 400 μl. Concentration of NaCl in the assay mixture was adjusted to 150 mM and antibody was added. The immune complexes were formed by incubation on ice for 2 h. To remove insoluble materials, each sample was spun for 10 min by a table-top centrifuge at 15,000 rpm, and the supernatant was transferred into a fresh tube. Thirty μl of Protein A sepharose beads (Pharmacia Biotech Inc., Uppsala, Sweden; 0.1 mg/ml in modified H buffer) were added followed by incubation for 2 h at 4°C with rocking. Immune complexes were centrifuged at 1,500 rpm for 30 s in a Sorvall table-top centrifuge prechilled to 4°C. Supernatant was discarded and the pellet was washed three times carefully with 1 ml of ice-cold modified H buffer containing 150 mM NaCl by inverting the tubes five times. Thirty μl of 2× Laemmli sample buffer (Sambrook *et al.*, 1989) was added to sepharose bead, and boiled 5 min before SDS PAGE. For kinase assay, beads were washed twice with modified H buffer containing 150 mM NaCl and twice with kinase buffer (Elion *et al.*, 1993). Kinase assay was performed in 30 ml reactions in kinase assay buffer containing 10 μM ATP and 1 μCi γ-[^{32}p]ATP. After 10 min incubation at 30°C, reactions were terminated by the addition of 30 μl of 2× Laemmli sample buffer. Samples were boiled for 5 min and separated by running 10% SDS PAGE. After electrophoresis, the gel was soaked for 1 h in a solution containing 10% acetic acid, 10% methanol, 0.5% phosphoric acid, and 10 mM KPO_4. The gel was further soaked in a solution containing 10% acetic acid and 10% methanol for 30 min before drying. Autoradiographs were obtained after exposure of the gel at −70°C for 8 h with an intensifier screen.

Results and Discussion

S. cerevisiae Ste5 is a scaffold protein of the pheromone pathway and it endows specificity for the pheromone pathway by recruiting many components of the signaling cascade including components for G1 arrest and mating. Ste5 interacts with Ste20, Ste11, Ste7, and Fus3 kinases in different proportions, and forms a high molecular weight complex in a glycerol gradient (Choi *et al.*, 1994; 1999a). Furthermore, Fus3 present in the high molecular weight complex fractions displays the highest kinase activity (Choi *et al.*, 1999a).

These results suggest that Ste5 plays an essential role in the formation of the high molecular weight complex and that the formation of the high molecular complex may be required for activation of Fus3 MAPK. It was suggested that Ste5 might endow specificity for the pheromone pathway by recruiting the pheromone signaling components. Therefore, components of pheromone pathways may be sequestered from other components of homologous MAPK pathways using similar or the same signaling components with the pheromone pathway. Ste5 is also involved in the activation of Fus3 MAPK, which is required for phosphorylation of Far1 for G1 arrest and Ste12 for mating (Kranz *et al.*, 1993; Peter *et al.*, 1993). Although Ste5 is involved in both G1 arrest and the mating process, it is not well known how Ste5 is involved in the regulation of Fus3 MAPK for G1 arrest and mating.

The *ste5*$^{\Delta\text{-}776}$ and *ste5M*^{D248V} transformants transmit theirs mating signals through Fus3 MAPK To better understand the roles of Ste5 in the activation of Fus3, we analyzed several G1 arrest defective Ste5 mutants retaining which mating abilities (*ste5*$^{\Delta\text{-}776}$ and *ste5M*^{D248V}) and a mutant which lost both G1 arrest and mating functions (*ste5M*^{Y421D}) (Choi *et al.*, 1999b). Isolation and characterization of these mutants suggested that the roles of Ste5 in G1 arrest and mating are separate, as well as the role of Ste5 in the differential activation of Fus3 for G1 arrest and mating (Choi *et al.*, 1999b). However, it is also possible that the mating signal of Ste5 mutants may use Kss1 rather than Fus3. It is known that Kss1 can replace Fus3 MAPK in mating, but not G1 arrest in the absence of Fus3 (Cairns *et al.*, 1992; Elion *et al.*, 1991). Because the *ste5*$^{\Delta\text{-}776}$ and *ste5M*^{D248V} mutants retain their mating function (Choi *et al.*, 1999b), it is possible that these mutants may use Kss1 for mating with defect to communicate with Fus3. In this case, the mating function of Ste5 mutants (*ste5*$^{\Delta\text{-}776}$ and *ste5M*^{D248V}) could be acquired through Kss1 with complete loss of the ability to communicate with Fus3. Identification of the source MAPK (either Fus3 or Kss1) for mating function of the *ste5*$^{\Delta\text{-}776}$ and *ste5M*^{D248V} mutants is important because we cannot postulate separate roles for Ste5 in G1 arrest and mating if the mating signal passes through Kss1 rather than Fus3.

To learn the source MAPK for mating, we transformed a *kss1Δ ste5Δ* double mutant (Elion *et al.*, 1991) with a plasmid containing gene for G1 arrest defective *ste5* mutant retaining mating capacity, pKC20M69 (*ste5*$^{\Delta\text{-}776}$ *URA3* 2μ) or pKC20M72 (*ste5M*^{D248V} *URA3* 2μ), respectively (Choi *et al.*, 1999b) and the mating capabilities of these transformants were checked by patch mating analysis (Sprague, 1991).

The *kss1Δ ste5Δ* strain mated well with mating partner cells when the cells transformed with pKC20 (*STE5M* 2μ), however, the cells did not mate by transformation of Yep24 vector alone (Fig. 1). Mating capacities of the *ste5M*^{D248V} or *ste5*$^{\Delta\text{-}776}$ transformants were similar to those of a *STE5M* transformant, therefore, those cells containing *ste5M*^{D248V} and *ste5*$^{\Delta\text{-}776}$ grew similarly on a diploid selective plate (Fig. 1). These results suggested that the mating signal of the *ste5M*^{D248V} and *ste5*$^{\Delta\text{-}776}$ mutants passed through Fus3 rather than Kss1. Therefore, our notion of separate roles of Ste5 in G1 arrest and mating (Choi *et al.*, 1999b) was appropriate.

Suppression patterns of G1 arrest defective *ste5M* mutants support a role of Ste5 as a scaffold protein To better understand the relationship between Ste5 and MAPK module kinases (Ste11, Ste7, and Fus3), we performed high copy suppressor analysis (Elion, 1995). In this epistasis experiment, we expected to learn the nature of *ste5* mutants and the functional location of Ste5 in the pheromone signaling cascade. Here, we over-expressed each *STE11*, *STE7*, *FUS3*, or *KSS1* gene in the transformants containing a plasmid for *ste5*$^{\Delta\text{-}776}$, *ste5M*^{D248V}, or *ste5M*^{Y421D} gene respectively, and their abilities to suppress G1 arrest function were checked by halo assay (Sprague, 1991). As shown in Fig. 2, suppression patterns of G1 arrest defective *ste5M* mutants by a component of pheromone pathway were variable.

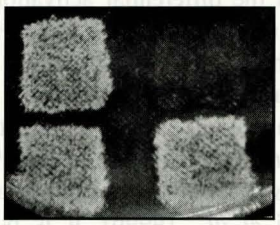

Fig. 1. Mating of Kss1 defective (*kss1Δ*) yeast strains harboring a G1 arrest defective *ste5M*. The pKC20 (*STE5M URA3* 2μ), Yep24 (2μ *URA3* vector), pKC20M72 (*ste5M*^{D248V} *URA3* 2μ)' or pKC20M69 (*ste5*$^{\Delta\text{-}776}$ *URA3* 2μ) plasmid was transformed into EKY431 (*MATα kss1Δ::TRP1 ste5Δ::HIS3*) yeast strain and transformants were selected on SC-ura plate. Mating capacities of the transformants with L1543 mating partner cells (*MATα lys9*) were performed by patch-mating analysis as described in Materials and Methods.

The *ste5*$^{\Delta\text{-}776}$ and *ste5M*^{D248V}mutants were suppressed by *STE11* and *STE7*, although not significantly by *FUS3/KSS1* (Fig. 2). However, suppression patterns of *ste5*$^{\Delta\text{-}776}$ or *ste5M*^{D248V} mutants by *STE11* and *STE7* were different. Although *ste5*$^{\Delta\text{-}776}$ mutant was suppressed significantly by *STE11*, it was only suppressed slightly by *STE7*. However, the *ste5M*^{D248V}mutant was suppressed more significantly by *STE7* than *STE11*. The *ste5M*^{Y421D} mutant was not suppressed by any of the genes we tested. Therefore, our G1 arrest *ste5M* mutants were suppressed allele specifically by overproduction of individual pheromone signaling components. Not all of the mutants were suppressed by high copy number *FUS3* or *KSS1* gene. These results suggested that suppression patterns of *ste5M* transformants demonstrate that the signal transduction involved in Ste5 is quite complex and it is difficult to explain by a simple linear signal transduction model (Elion, 1990; Gartner *et al.*, 1992; Sprague and Thorner, 1993), and it supports a model of pheromone signal transduction involving a scaffold protein, Ste5 (Choi *et al.*, 1994; Elion *et al.*, 1995; Herskowitz, 1995). Our results were in agreement with previous results of epistasis analysis of Ste5 which showed inconsistent suppression patterns dependent on different experimental approaches (Gartner *et al.*, 1992; Hasson *et al.*, 1994; Leberer *et al.*, 1993; Stevenson *et al.*, 1992). Due to the broad functioning position, the scaffold protein, Ste5, cannot be localized on a specific location in a position of the pheromone signaling cascade.

Interaction of G1 arrest defective Ste5M proteins with Fus3 Since the mutation site of *ste5M*^{D248V} is located in a region which might interact with Fus3 (Choi *et al.*, 1994; 1999b), we tested whether our Ste5M mutant proteins could interact with Fus3 by co-immunoprecipitation studies. First, cell extracts were prepared from different *ste5M* transformants, and the Ste5M proteins were detected by Western blot analyses using anti-Myc antibody. All the transformants except *ste5*$^{\Delta\text{-}776}$ transformant produced equivalent levels of Ste5M proteins (Fig. 3A). The Ste5$^{\Delta\text{-}776}$ protein was not detected by Myc antibody, due to the loss of *MYC* from part of the *ste5M* gene (Choi *et al.*, 1999b). The level of Ste5M proteins was not affected by treatment with α-factor. Since the levels of Ste5M proteins are similar, we purified the Ste5M proteins to determine the levels of co-purified Fus3. The levels of the copurified Fus3 in the anti-Myc immune precipitate were checked by Western blot analysis using Fus3 polyclonal antibody. When Ste5M^{D248V} and Ste5M^{Y421D} proteins were immunopurified by Myc antibody, the Fus3 was also co-purified as in the case of wild type Ste5M (Fig. 3B). The Ste5$^{\Delta\text{-}776}$ protein did not co-purify Fus3 because the protein itself cannot be purified by Myc antibody. Therefore, we do not know whether the Ste5$^{\Delta\text{-}776}$ interacts with Fus3 or not. Co-purification of Fus3 with the Ste5M^{D248V} *in vitro* suggests

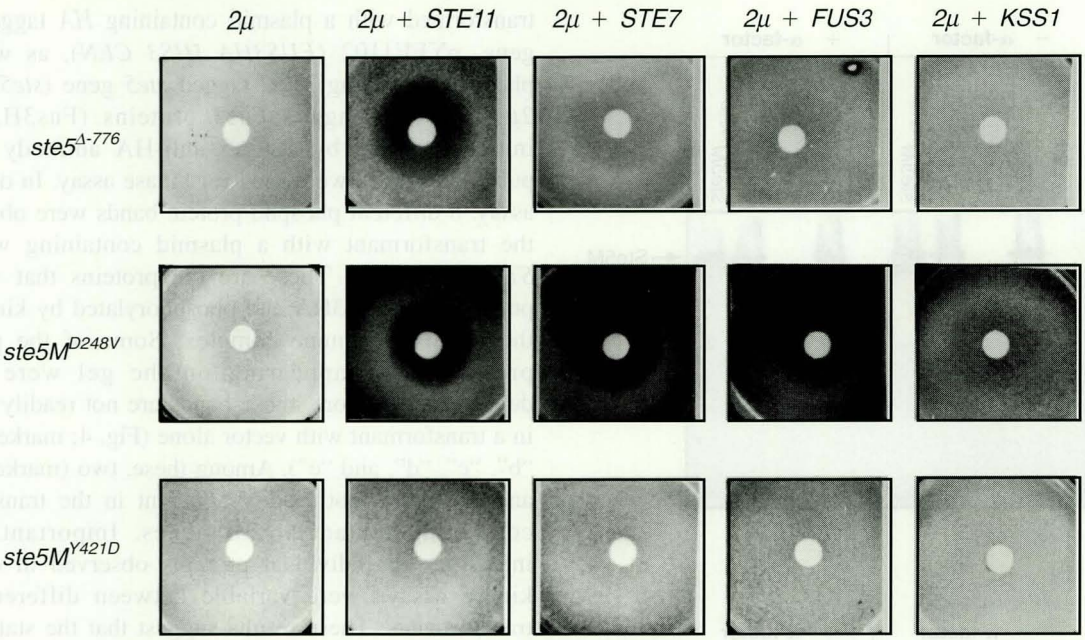

Fig. 2. Suppression of the G1 arrest defective *ste5M* transformants by overexpression of pheromone signaling components. The individual gene of pheromone signaling components in a 2μ vector (pSTE11.1 for *STE11*, pSTE7.4 for *STE7*, pKC29 for *FUS3*, or pXT1 for *KSS1*) was transformed into EKY150 yeast cells (*ste5Δ::TRP1*) containing pKC20M69 ($ste5^{\Delta\text{-}776}$), pKC20M72 ($ste5M^{D248V}$), or pKC20M105 ($ste5M^{Y421D}$) plasmid, respectively. Halo assays of the transformants were performed as described in Materials and Methods.

that Asp-248 of Ste5 protein may not be critical for Fus3 binding. Compared to the wild type Ste5M, $Ste5M^{D248V}$ and $Ste5M^{Y421D}$ mutant proteins increased levels of Fus3 protein co-purified in the cells not treated with α-factor. However, the level of Fus3 protein co-purified with Ste5M is higher than the levels of Fus3 protein co-purified by $Ste5M^{D248V}$ and $Ste5M^{Y421D}$ mutants in the cells treated with α-factor. Increases in the levels of Fus3 protein co-purified by $Ste5M^{D248V}$ and $Ste5M^{Y421D}$ mutant proteins suggest that the Fus3 binding affinities of the mutants somehow probably increased due to structural changes in the resting cells. It is more likely that the wild type Ste5M protein in the resting cells (not treated with α-factor) may have a protein structure that prohibits interaction with the substrate, Fus3, and structural change by Asp-248 or Tyr-421 mutation may disrupt a structure, which can limit binding of Fus3 in the resting cells. No change in the levels of Fus3 protein co-purified with $Ste5M^{D248V}$ and $Ste5M^{Y421D}$, regardless of α-factor treatment, suggests that the mutant proteins may have lost their ability of α-factor dependent structural inter-conversion for substrate binding.

α-Factor dependent induction of Fus3 requires intact Ste5M The high level of Fus3 protein co-purified by wild type Ste5M protein in the cells treated with α-factor could also be due to the high level of endogenous Fus3 protein in the wild type *STE5M* transformant. Therefore, endogenous Fus3 levels in different *STE5M* or *ste5M* transformants were probed by using Western blot analysis using crude cell extracts (Fig. 3B; middle panel). The level of endogenous Fus3 increased 2– to 3-fold after treatment with α-factor in wild type *STE5M* transformant (Fig. 3B; middle panel, compare lane 2 with 7). However, transformants containing the $ste5M^{D248V}$ or $ste5M^{Y421D}$ gene did not change the endogenous Fus3 levels after treatment with α-factor. The levels of a control protein, tublin, were constant within all the transformants regardless of α-factor treatment. Therefore, high levels of Fus3 protein copurified by wild type Ste5M protein, may be attributed to an increase in the level of Fus3 protein as well as conversion of Ste5 protein favorable for Fus3 binding in the cells containing wild type *STE5M*. The α-factor dependent induction of Fus3 mRNA was also observed in a previous study (Elion *et al.*, 1990). Since our transformants with a mutant *ste5M* gene did not increase Fus3 levels after treatment with α-factor, induction of the Fus3 protein may require Ste5M. Therefore, blocking signal transduction by *ste5M* mutations may interrupt the positive loop for the induction of Fus3.

Ste5 may be a determining factor for substrate specificity of Fus3 MAPK *in vivo* To understand the roles of Ste5 in the activation of Fus3 MAPK, Fus3 kinase assays were performed with cell extracts obtained from the transformants containing a different *ste5M* gene. For kinase assay, *ste5Δ fus3Δ* yeast double mutants were

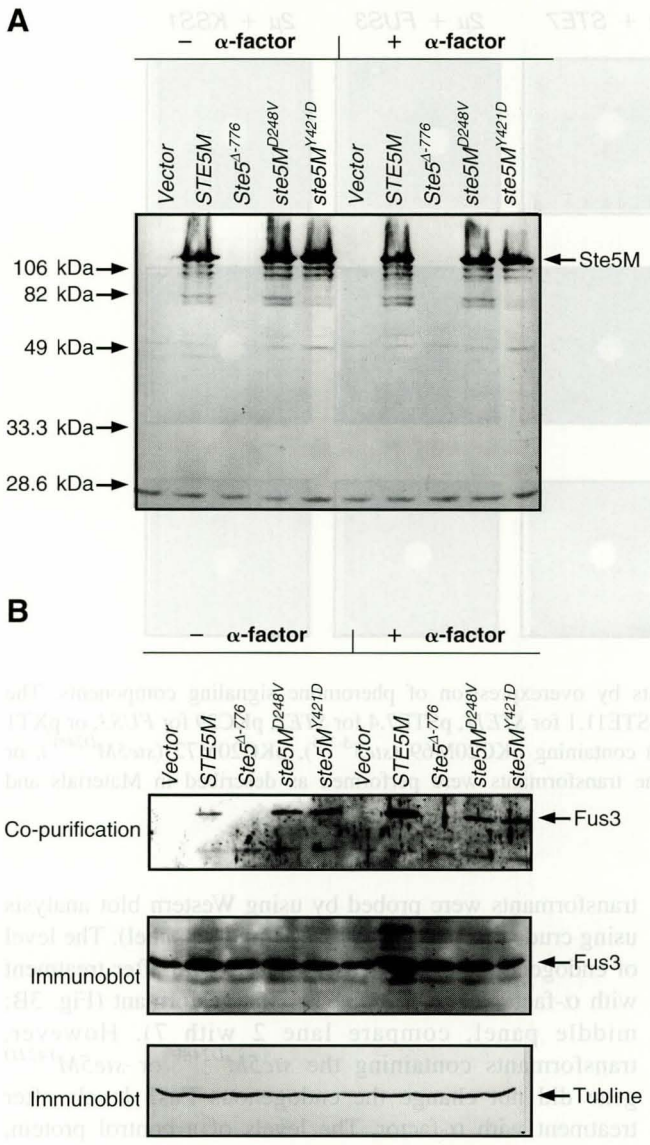

Fig. 3. Expression Ste5M proteins and co-purification of Fus3 by the G1 arrest defective Ste5M mutant proteins. Host EY150 (*ste5Δ::TRP1*) was transformed by a plasmid with different *ste5M* gene: lanes 1 and 6, Yep24 (2μ vector); lanes 2 and 7, pKC20 (*STE5M* 2μ); lanes 3 and 8, pKC20M69 ($ste5^{\Delta\text{-}776}$ 2μ); lanes 4 and 9, pKC20M72 ($ste5M^{D248V}$ 2μ) ; lanes 5 and 10, pKC20M105 ($ste5M^{Y421D}$ 2μ). Extracts were made from cells non-induced (minus α-factor) or induced for 1 h with 50 nM α-factor (plus α-factor). **A.** For detection of Ste5M proteins, 100 μg of each whole cell extract was separated by 10% polyacrylamide gel, and the Ste5M proteins were detected by Western blot analysis using Myc antibody. **B.** Each Myc epitope tagged Ste5 protein in the crude extracts (each 500 μg) was immunopurified with 5 μg of 9E10 anti-Myc monoclonal antibody, and co-purified Fus3 proteins were detected by Western blot analysis using Fus3 anti-rabbit polyclonal antibody (upper panel). Endogenous Fus3 and tubline were detected by Western blot analysis of 100 μg total proteins separated by 10% SDS PAGE (middle panel). Endogeneous levels of tubline were detected by anti-α-tubline antibody.

transformed with a plasmid containing *HA* tagged *FUS3* gene, pYEE1102 (*FUS3HA HIS3 CEN*), as well as a plasmid containing *MYC* tagged *ste5* gene (*ste5M URA3* 2μ). The HA tagged Fus3 proteins (Fus3HA) were immunopurified by 12CA5 anti-HA antibody and the purified samples were used for kinase assay. In our kinase assay, 8 different phospho-protein bands were observed in the transformant with a plasmid containing wild type *STE5M* (Fig. 4). These are the proteins that were co-purified with Fus3HA and phosphorylated by kinase(s) in the Fus3HA immune complex. Some of the phospho-protein bands appearing on the gel were *STE5M* dependent, therefore, those bands are not readily apparent in a transformant with vector alone (Fig. 4; marked by "a", "b", "c", "d", and "e"). Among these, two (marked by "b" and "e") were not readily apparent in the transformants containing mutant *ste5M* genes. Importantly, band intensities of individual proteins observed in our Fus3 kinase assays were variable between different *ste5M* transformants. These results suggest that the status of the Ste5 protein may be essential for interaction and phosphorylation of these proteins. Those phospho-proteins may interact directly with Fus3HA or indirectly by interacting with the protein(s) associated with Fus3HA, like Ste5 (Choi *et al.*, 1994; Elion *et al.*, 1993; Kranz *et al.*, 1994). Previous studies showed that Ste5 interacted with Ste20, Ste11, Ste7, Fus3/Kss1 kinases as well as Bem1 and β subunit of G protein (Brill *et al.*, 1994; Choi *et al.*, 1994; Elion *et al.*, 1993; Errede *et al.*, 1993; Kranz *et al.*, 1994; Zhou *et al.*, 1993). Phospho-protein bands uniquely observed in the transformant with wild type *STE5M* could be one of those proteins associated with Ste5. The phospho-protein bands marked by "a", "b" and "d" arrows are similar to the size of Ste5M (120 kDa), Far1 (110 kDa)/Ste12 (106 kDa)/Ste20 (102 kDa), and Ste7 (65 kDa) (Elion *et al.*, 1993; Leeuw *et al.*, 1995). A phospho-protein band, marked by an "e" arrow which was uniquely observed in the wild type *STE5M* transformant, could be 42 kDa Fus3HA protein. A previous study showed that Fus3 can be autophosphorylated. However, auto-phosphorylation activity was not observed in a *ste5Δ* strain (Gartener *et al.*, 1992). The three phospho-proteins that appeared as bands in the transformant containing vector alone may also interact with Fus3, but its interaction may be independent from Ste5. However, Ste5 may still be required for optimum phosphorylation of these proteins due to attenuated band intensities of those three phosho-proteins in a transformant containing vector alone compared with other transformants. Since two phosphoproteins bands ("b" and "e") are not readily apparent in our *ste5M* mutants, phosphorylation of those bands may be required for G1 cell cycle arrest, but may not play a critical role in mating. Differential patterns and intensities of the protein phosphorylation in the Fus3 immune complex were obtained by different genetic status

of Ste5. These results suggest that Ste5 may be important for discrimination of substrate specificity of Fus3. Although differences in the band intensities were observed, we did not observe any difference in the patterns of phospho-protein bands between mating-efficient and mating-defective *ste5M* mutants in our kinase assay.

There is a possibility that phospho-proteins observed in our kinase assay may also be phosphorylated by kinase(s) associated with Fus3HA (such as *STE11* and *STE7*) rather than Fus3HA itself. Therefore, we performed Fus3 kinase assay by supplying a Fus3 substrate, myelin basic protein (MBP), to probe for unique Fus3 kinase activity. Although MBP was specifically phosphorylated in a cell extract from a transformant containing wild type *STE5M*, it was not phsphorylated in the transformants containing a mutated *ste5M* or a transformant containing a vector alone (Fig. 4; lower panel). Therefore, wild type Ste5M may be required for specific activation of Fus3 kinase. This results agree with the fact that phosphorylation of 42 kDa substrate (possible Fus3 MAPK) was only observed in the transformant containing wild type *STE5M*. Since MBP was not significantly phosphorylated in mating-efficient $ste5^{\Delta\text{-}776}$ and $ste5M^{D248V}$ transformants, the Fus3 kinase activity observed in our kinase assay may not be a probe for signal transduction required for mating, although it does for G1 cell cycle arrest. Again, the levels of Fus3HA proteins were similar in our kinase assay mixtures, therefore, the difference of Fus3HA activity may not be due to the differences in the protein levels. Although levels of phospho proteins were variable, we could not detect any significant differences in the phosphorylation patterns of proteins between mating-efficient and mating-defective G1 arrest *ste5M* mutants. Therefore, our kinase assay system may not sensitively detect the differences between mating-efficient and mating-defective G1 arrest defective *ste5M* mutants. A previous study showed that Ste5 might have different roles in G1 arrest and mating by genetic studies (Choi *et al.*, 1999b). In this study, we provided biochemical evidence for the different roles of Ste5 in G1 arrest and mating. In addition, our studies revealed that Ste5 might play a role in the induction of Fus3. Furthermore, we suggest that Ste5 is a factor in the selection of substrate specificity required for G1 arrest or mating.

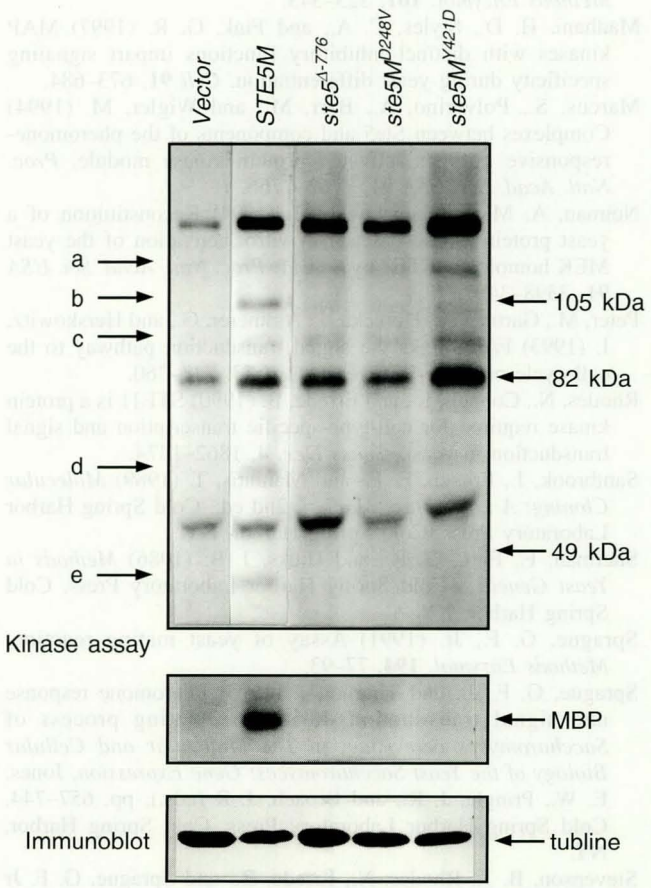

Fig. 4. Fus3HA immune kinase activities in transformants containing different Ste5M proteins. The EKY174 (*fus3Δ ste5Δ*) yeast cells were transformed by pYEE1102 (a plasmid containing *FUS3HA*) and a plasmid with Yep24 (vector), pKC20 (*STE5M*), pKC20M69 ($ste5^{\Delta\text{-}776}$), pKC20M72 ($ste5M^{D248V}$), or pKC20M105 ($ste5M^{Y421D}$) respectively, and the transformants were selected on SC-Ura His plates. Extracts were made from the transformants induced for 1 h with 50 nM α-factor. The Fus3HA protein was immuno-purified by 12CA5 anti-HA antibody from 400 μg of cell extracts prepared from α-factor induced cells, and kinase assay of each immunoprecipitate was performed either with or without exogenous substrate, MBP, as described in Materials and Methods. Upper panel represents phosphorylation patterns of endogenous proteins associated with Fus3. The *STE5M* dependent phospho-proteins observed in the Fus3HA immune complex(es) are marked by arrows. The lower panel represents phosphorylation patterns of exogenous substrate, MBP.

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