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Ste5 RING-H2 Domain: Role in Ste4-Promoted Oligomerization for Yeast Pheromone Signaling

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Ste5 is a scaffold for the mitogen-activated protein kinase (MAPK) cascade components in a yeast pheromone response pathway. Ste5 also associates with Ste4, the β subunit of a heterotrimeric guanine nucleotide–binding protein, potentially linking receptor activation to stimulation of the MAPK cascade. A RING-H2 motif at the Ste5 amino terminus is apparently essential for function because Ste5(C177S) and Ste5(C177A C180A) mutants did not rescue the mating defect of a *ste5*Δ cell. In vitro Ste5(C177A C180A) bound each component of the MAPK cascade, but not Ste4. Unlike wild-type Ste5, the mutant did not appear to oligomerize; however, when fused to a heterologous dimerization domain (glutathione S-transferase), the chimeric protein restored mating in an *ste5*Δ cell and an *ste4*Δ *ste5*Δ double mutant. Thus, the RING-H2 domain mediates Ste4-Ste5 interaction, which is a prerequisite for Ste5-Ste5 self-association and signaling.

Mating in the yeast *Saccharomyces cerevisiae* is initiated by pheromone binding to heterotrimeric guanine nucleotide–binding protein (G protein)–coupled receptors on cells of opposite mating type, leading to dissociation of the Gβγ complex (Ste4 Ste18) from the inhibitory Gα (Gpa1) subunit (*1*). Subsequent signal propagation activates an evolutionarily conserved MAPK cascade (*2*), ultimately causing arrest of the cell cycle in the G_1 phase and the production and activation of factors required for cell and nuclear fusion. Ste5 is an essential component of this pathway (*3*, *4*) and is thought to function as a scaffold for the MAPK cascade components (*5*). Ste5 also associates with Ste4 (*6*) and thus may link release of Gβγ to activation of the MAPK cascade.

The NH_2-terminus of Ste5 (residues 177 to 229) contains a cysteine-rich region that is the prototype for the RING-H2 motif (Fig. 1A). The RING-H2 motif is a variant of the larger class of RING domains (*7*) but contains a second histidine in place of the cysteine normally found at position 5 (Fig. 1B). Proteins possessing RING and RING-H2 domains participate in diverse cellular processes, but no specific function has yet been ascribed to these domains (*7*). The crystal structures of two RING domains have been solved (*8*), and each is a globular pseudosymmetric fold that coordinates two Zn^{2+} atoms through a cross-bridging element. To disrupt this structure as a means to determine its importance to the function of Ste5, we mutated [to serine (S) or alanine (A), as indicated] either the first, or both the first and second, conserved cysteine (C), yielding $Cys^{177} \rightarrow Ser^{177}$ (C177S) and C177A C180A mutant proteins. The expressed amount and stability of both mutant proteins were comparable to those of wild-type Ste5 (*9*).

Neither the *ste5(C177S)* allele nor the *ste5(C177A C180A)* allele was able to complement the mating defect of an *ste5*Δ strain, when the mutant genes were expressed from a centromere-based plasmid, driven by either the *STE5* promoter or the inducible *GAL1* promoter (Table 1). Even high-level expression of the mutant proteins from the *GAL1* promoter on a multicopy plasmid caused only a small increase in the mating proficiency of the *ste5*Δ cells (Table 1). Thus, an intact RING-H2 domain is essential for Ste5 function. Consistent with this observation, *ste5*Δ cells containing the *ste5(C177A C180A)* allele were unable to activate transcription from a pheromone-inducible reporter gene (*FUS1-lacZ*) (*10*) in response to the mating pheromone α-factor (*9*). Likewise, *ste5*Δ cells expressing the Ste5(C177A C180A) mutant were unable to respond to α-factor (*9*), as judged by the halo bioassay for pheromone-induced growth arrest (*11*).

When Ste5(C177A C180A) was overexpressed from the *GAL1* promoter on a multicopy plasmid in a $STE5^+$ strain, the cells formed a halo in response to α-factor, but the halo filled in much more rapidly than did lawns of control cells lacking the plasmid (*9*), indicating an attenuated G_1 arrest response in these cells. Such a dominant-negative effect might result if the mutant protein competed with wild-type Ste5 for the binding of one or more of the factors required for signaling and sequestered them in an inactive complex. To determine directly whether the Ste5(C177A C180A) mutant protein remained competent to interact with any of the known MAPK cascade components, we immunoprecipitated extracts from cells expressing derivatives of Ste5 and Ste5(C177A C180A), tagged at their NH_2-terminal end with a c-Myc epitope, with an appropriate mono-

A

Protein	Organism	RING-H2 Domain Sequence (177 → 229)
Ste5	Yeast	**C**TL**C**DEPISNRRKGEKIIEL**AC**G**H**L**S**H**Q**E**C**LIISFGTTSKADVRALFPF**C**T**KC**
Deltex	*Drosophila*	**C**PM**C**MEELVHSAQNPAISLSR**C**Q**H**LM**H**LQ**C**LNGMIIAQQNEMN**K**NLFIE**C**PV**C**
Far1	Yeast	**C**LIC**E**ESISSTFTGEKVVEST**C**S**H**TS**H**YN**C**YLMLFETLYFQ GKFP**E**C**K**I**C**
Rapsyn	Human	**CA**LC**G**ESIGE KNSRLQALP**C**S**H**IF**H**LR**C**LQN NGTR**S**C**P**N**C**
Neurodap1	Rat	**C**PIC**C**SEYI KDDIATELP**C**H**H**FF**H**KP**C**VSIWL QKSGT**C**P**VC**
Pep3	Yeast	**C**DE**C**GKFL QIKKFIVFP**C**G**H**CF**H**WN**C**IIRVILNSNDY(24)IVE**K**C**G**L**C**
Pep5	Yeast	**C**FM**C**RLTL DIPVVFFK**C**G**H**IY**H**Q**H**CLNEEEDTLESE RKLFK**C**P**K**C

B Consensus **-Cys-X_2-Cys-X_{12-17}-Cys-X-His-X_2-His-X_2-Cys-X_{8-39}-Cys-X_2-Cys-**

Fig. 1. Sequence alignment and derived consensus for the RING-H2 motif. (**A**) Shown are RING-H2 domains of the following proteins (with the indicated GenBank accession numbers): Ste5 (L23856); Deltex (U09789); Far1 (M60071); Rapsyn (Z33905); Neurodap1 (D32249); Pep3 (M65244); and Pep5 (X54466). Conserved residues are indicated in bold and represent presumptive Zn^{2+}-binding ligands. (**B**) Consensus sequence for the RING-H2 motif. Positions conserved in all members are given in bold. X represents any amino acid, and the number of such residues is also indicated.

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clonal antibody (9E10) (*12*). The immune complexes were washed, and the bound proteins were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a membrane filter for immunoblotting. The filters were probed with polyclonal antisera to the MAPK Fus3, the MAPK kinase Ste7, and the MAPK kinase kinase Ste11. Ste5(C177A C180A) associated with Ste11, Ste7, and Fus3 with an affinity virtually indistinguishable from that of wild-type Ste5 (Fig. 2, A through C), indicating that disruption of the RING-H2 domain does not prevent binding of the MAPK cascade components and, therefore, does not compromise the overall structure of the Ste5 protein.

Because Ste5 can associate with Ste4 (Gβ subunit), and this interaction requires the NH_2-terminal region of Ste5 (6), we examined the effect of the RING-H2 domain point mutations on the ability of Ste5 to interact with Ste4. Ste4 was not immunoprecipitated with the Ste5(C177A C180A) mutant, whereas it was with wild-type Ste5 (Fig. 2D). Thus, when the RING-H2 domain is disrupted, interaction of Ste5 with Ste4 is severely diminished. We confirmed that the RING-H2 domain is essential for Ste4 association with Ste5 in the two-hybrid system (6, *13*). Both the *ste5(C177S)* and *ste5(C177A C180A)* alleles (as well as Ste5 mutants with deletions of the sequences encompassing the RING-H2 domain) failed to interact with Ste4, but still interacted with all three of the MAPK components, whereas normal Ste5 interacted with all four partners (*9*).

Ste5 can also self-associate (*14*, *15*). To test whether the Ste5(C177A C180A) mutant could oligomerize, we performed interallelic complementation tests. We used *ste5* point mutations that result in a specific defect in the association of Ste5 with one, and only one, component of the MAPK cascade (*15*). Neither Ste5(I504T), which does not interact with Ste11, nor Ste5(V763A S861P), which fails to interact with Ste7, when expressed alone, complemented a *ste5Δ* cell, whereas normal Ste5 expressed from the same vector did (Fig. 3). However, expression of both mutant proteins in the same cell restored efficient mating (Fig. 3), suggesting that Ste5 action in vivo requires oligomer formation (*14*, *15*). In contrast, the *ste5(C177A C180A)* allele was unable to rescue the mating debility of *ste5Δ* cells when coexpressed with either the *ste5(V763A S861P)* allele (Fig. 3) or the *ste5(F514L)* allele (*9*), despite

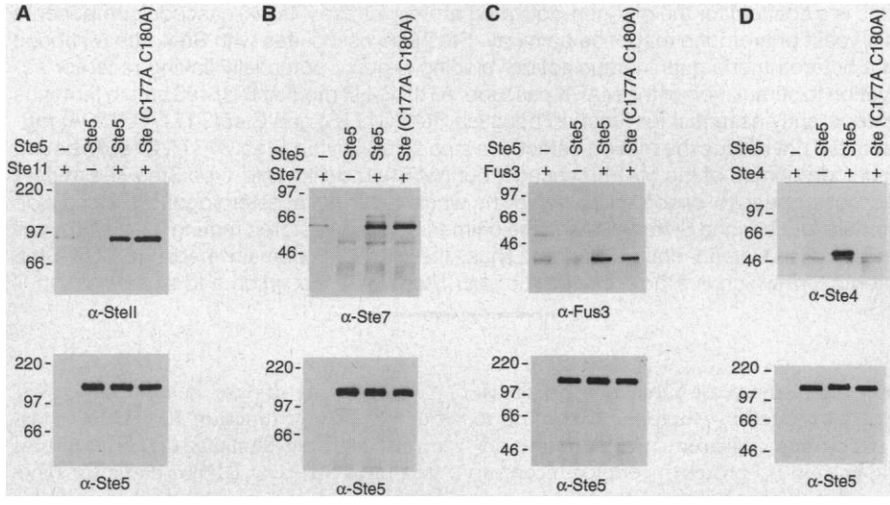

Fig. 2. Binding of Ste5(C177A C180A) to Ste11, Ste7, and Fus3, but not Ste4. A protease-deficient strain BYB84 (*MAT***a** *ste5Δ*) (*21*) carrying a vector alone (–) or the same plasmid expressing either Myc-tagged Ste5 or Myc-tagged Ste5(C177A C180A) (*22*) was cotransformed with either another empty vector or the same vector expressing either Ste11 (**A**), Ste7 (**B**), Fus3 (**C**), or Ste4 (**D**). The cells were grown, induced for protein expression, lysed, and subjected to immunoprecipitation with an anti-Myc monoclonal antibody 9E10 (*12*). The resulting immune complexes were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by immunoblotting (*24*) with rabbit polyclonal antibodies specific for Ste11 (A, top), Ste7 (B, top), Fus3 (C, top), Ste4 (D, top) or Ste5 [(A) through (D), bottom]. Despite overexpression, all of the untagged proteins displayed only weak nonspecific adsorption to the 9E10 antibody. Results shown are representative of at least three different experiments, each performed with independent transformants. Molecular size markers are in kD.

Table 1. Quantitative mating assays. Strain BYB69 (*MAT***a** *ste5Δ*) (*21*) was transformed with appropriate vector controls (YCplac33, YCpUGal, or YEp352Gal) or with the derived plasmids indicated (*22*) expressing either wild-type *STE5*$^+$ or each of two different RING-H2 domain alleles: the single mutant *ste5(C177S)* or the double mutant *ste5(C177A C180A)*. The mating ability of the resulting transformants was tested, using minor modifications (*27*) of a quantitative procedure (*11*), with strain DC17 as the *MATα* partner. Values given represent the averages (and standard deviations of those means) for at least three independent trials, each performed in triplicate. ND, not determined.

Plasmid	Mating efficiency
YCplac33	$<10^{-6}$
YCp-*STE5*$^+$	0.44 ± 0.06
YCp-*ste5(C177S)*	$<10^{-6}$
YCp-*ste5(C177A C180A)*	$<10^{-6}$
YCpU*Gal*	$<10^{-6}$
YCp*Gal-STE5*$^+$	0.58 ± 0.08
YCp*Gal-ste5(C177S)*	ND
YCp*Gal-ste5(C177A C180A)*	$<10^{-6}$
YEp352*Gal*	$<10^{-6}$
YEp*Gal-STE5*$^+$	0.34 ± 0.1
YEp*Gal-ste5(C177S)*	1.3×10^{-3} $\pm 6.5 \times 10^{-4}$
YEp*Gal-ste5(C177A C180A)*	2.2×10^{-4} $\pm 5.1 \times 10^{-5}$

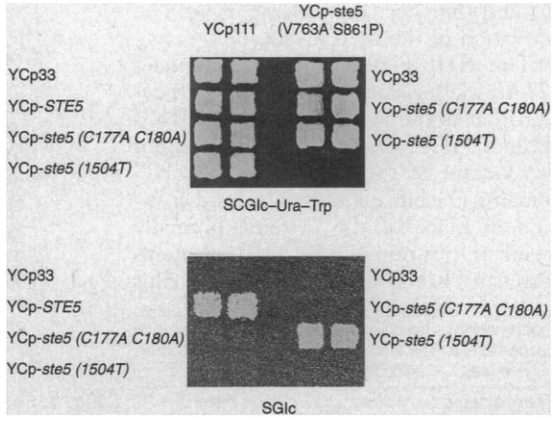

Fig. 3. Genetic analysis of Ste5-Ste5 interaction by interallelic complementation. Strain BYB69 (*MAT***a** *ste5Δ*) (*21*) was transformed with either vector alone (YCp111) or the same vector expressing the *ste5(V763A S861P)* mutant allele, which is defective for interaction with Ste7 (*15*). These two derivatives were then cotransformed with either a vector control (YCp33) or with the same vector expressing either wild-type Ste5 or each of two different *ste5* mutants: the RING-H2 domain mutant, *ste5(C177A C180A)*; or, the *ste5(I504T)* allele, the latter of which is defective for interaction with Ste11 (*15*). The resulting plasmid-bearing strains were patched onto selective plates lacking uracil and tryptophan (upper panel), mated to an appropriate *MATα* tester strain (DC17), and subsequently replica-plated onto a medium selective for diploids (lower panel) (*25*). Patches from two independent transformants containing each combination of plasmids are shown.

the fact that the RING-H2 domain mutant is only defective in its ability to associate with Ste4. These results suggest that, in addition to its role in mediating Ste5 association with Ste4, the RING-H2 domain is also required for Ste5 oligomerization.

We tested whether fusion of the Ste5(C177A C180A) protein to a heterologous dimerization domain would allow the mutant protein to function. For this purpose, we used *Schistosoma japonicum* glutathione-S-transferase (GST), which forms a stable dimer, both in solution and in protein crystals (*16*), and which can functionally substitute for the dimerization domain of a heterologous protein (*17*). Indeed, a chimeric form of Ste5(C177A C180A), in which GST was fused to the COOH-terminus (at residue 913), did enable *ste5*Δ cells to mate, and did so as well as a fusion of GST to wild-type Ste5 (Fig. 4A), within the limits of resolution of this assay (*15*). Also, expression of either Ste5-GST or Ste5(C177A C180A)-GST in *ste5*Δ cells induced *FUS1-lacZ* transcription (9). Thus, bringing Ste5(C177A C180A) molecules into proximity with each other by GST-mediated dimerization was sufficient to restore function to this mutant.

In immunoprecipitation experiments, the Ste5(C177A C180A)-GST chimera did not associate with Ste4 (9), indicating that GST-mediated dimerization of Ste5 did not reconstitute a binding site for Ste4. Because the chimera is functional, this observation implies that the normal order of events in pheromone-induced activation of Ste5 is association with Ste4, followed by oligomerization. If so, expression of the Ste5(C177A C180A)-GST chimera should bypass the need for Ste4, as well as the need for Ste5. Indeed, as predicted on the basis of our findings, a *ste4*Δ *ste5*Δ double mutant expressing the Ste5(C177A C180A)-GST fusion was able to mate (Fig. 4B), although not quite as well as cells expressing both wild-type *STE5* and *STE4*. Thus, even though Ste5(C177A C180A) cannot interact with Ste4 and cannot self-associate, once dimerized by fusion to GST, the resulting chimera is competent for signaling.

Expression of the wild-type Ste5-GST fusion, which supported mating of *ste5*Δ cells (Fig. 4A), did not rescue the mating defect of the *ste4*Δ *ste5*Δ double mutant (Fig. 4B). We conclude from this finding that the intact RING-H2 domain of wild-type Ste5 may have an inhibitory function that can only be alleviated upon its interaction with Ste4. The C177A C180A mutations, by perturbing the RING-H2 domain, apparently eliminate this negative function, such that artificial dimerization can promote signaling even in a cell that lacks Ste4. Presumably, therefore, in normal cells, interaction of Ste4 with Ste5 relieves the inhibitory function of the RING-H2 domain as well as promotes a conformational change that permits dimerization of Ste5, in that order.

Disruption of the RING-H2 domain in Ste5 by mutation of conserved cysteines abolished the function of Ste5 in mating. Likewise, naturally occurring point mutations in the RING element of the human breast cancer susceptibility–determining protein, BRCA1 (*18*), ablate its tumor-suppressor function. Our results indicate that association with the components of the MAPK cascade is not sufficient for Ste5 action. The RING-H2 domain is not only required for Ste4 binding to Ste5, but also (directly or indirectly) for Ste5 oligomerization. Thus, the RING-H2 domain of Ste5 serves as a molecular link between G protein activation and stimulation of the MAPK cascade. These observations raise the possibility that RING-H2 and RING domains in other proteins may mediate their multimerization or their association with (and regulation by) Gβγ subunits, or both. Finally, our evidence indicates that the RING-H2 domain in wild-type Ste5 also acts as a negative regulatory element. This inhibitory role may involve the proline-rich regions (residues 1 through 162 and 260 through 337) that immediately flank the RING-H2 domain on either side. In this regard, previous results (*4*) have shown that a mutation (T52M) in the region NH_2-terminal to the RING-H2 motif confers a partial, constitutively hyperactive phenotype, suggesting that this alteration perturbs the inhibitory function of the RING-H2 domain.

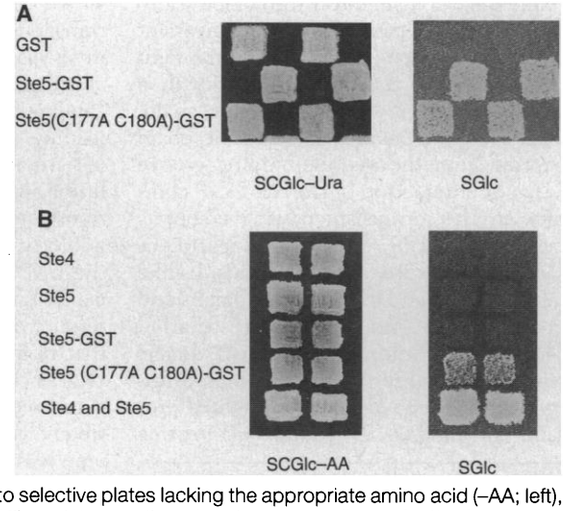

Fig. 4. Dimerization of the RING-H2 domain mutant restored Ste5 function and bypassed the requirement for Ste4. (**A**) Strain BYB69 (*MAT***a** *ste5*Δ) (*21*) transformed with a plasmid expressing GST alone, or either a Ste5-GST or a Ste5(C177A C180A)-GST chimera (*26*), were patched onto selective plates (left), mated to a *MAT*α tester strain (DC17), and subsequently replica-plated onto a medium selective for diploids (right). Patches from two independent transformants containing each plasmid are shown (*25*). (**B**) Strain BYB88 (*MAT***a** *ste4*Δ *ste5*Δ) transformed with a plasmid expressing Ste4 alone, Ste5 alone, the Ste5-GST fusion, the Ste5(C177A C180A)-GST fusion, or both Ste4 and Ste5 together, were patched onto selective plates lacking the appropriate amino acid (–AA; left), mated to the *MAT*α tester strain (DC17), and then replica-plated onto a medium selective for diploids (right). Patches from two independent transformants containing each plasmid, or combination of plasmids, are shown (*25*).

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21. Yeast strains used were BYB69 (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 lys2Δ::hisG trp1-1 ura3-1 ste5Δ::LYS2*), BYB84 (*MAT***a** *gal2 leu2 prb1-1122 pep4-3 prc1-407 trp1 ura3-52 ste5Δ*), BYB88 (*MAT***a** *ade2-101^{oc} his3-Δ200 leu2Δ-1 lys2-801^{am} trp1-Δ63 ura3-52 ste4Δ::TRP1 ste5Δ::LYS2*), and DC17 (MATα *his1*).
22. The *CEN* plasmid (pCJ117) expressing $(His)_6$- and Myc epitope–tagged *STE5*$^+$ from the *GAL1* promoter is described in detail elsewhere (*15*). A multicopy plasmid (pCJ6) expressing the same construct was produced by inserting the 3.3-kb Bam HI–Bam HI *STE5*-containing fragment from pCJ117 into the Bam HI site in the vector, YEp352Gal (*19*). pCJ93 expresses the same construct from the authentic *STE5* promoter in the vector, YCplac33 (*20*), and was engineered with polymerase chain reaction (PCR) to have the sequence, 5′-CATATGATG-3′, immediately upstream and in-frame with the first codon of the $(His)_6$ tag. The double mutant allele, *ste5(C177A C180A)*, used in most of the experiments presented here was produced by PCR amplification using a primer encoding the sequence 5′-AA**C**-GC**G**TCTGC-TACGTTAGCT-3′, in which the indicated bases (underlined) were altered to convert the Cys codons at positions 177 and 180 to Ala codons (and in which the silent mutations, indicated by boldface, were introduced to create an Mlu I site). The mutated segment was then used to replace the corresponding fragment in the other *STE5*-containing plasmids to generate constructs in which Ste5(C177A C180A) was expressed from the *STE5* promoter on a *CEN* plasmid (pCJ70), from the *GAL1* promoter on a *CEN* plasmid (pCJ119), and from the *GAL1* promoter on a multicopy plasmid (pCJ48).
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24. Protease-deficient strain BYB84 (*21*), carrying vector alone or expressing from the *GAL1* promoter NH_2-terminally c-Myc–tagged versions of either normal Ste5 or the RING-H2 domain mutant (*22*), and also carrying either a *CEN* vector alone, or the same vector expressing *STE11*, *STE7*, or *STE4* from the *GAL1* promoter, or expressing *FUS3* from the endogenous *FUS3* promoter on a 2 μm DNA-based vector, were grown under selection in SCRaf medium (*15*) to $A_{600\ nm}$ = 0.6. The cultures were induced by the addition of Gal to a final concentration of 2% and incubated for an additional 2 to 3 hours. The cells were collected, lysed, clarified, and the resulting extracts were subjected to immunoprecipitation (*15*). The resulting immune complexes were resuspended in 1× SDS-PAGE sample buffer, boiled for 5 min, and then resolved by SDS-PAGE. After transfer onto Immobilon-P membranes (Millipore) using semidry transfer apparatus (Bio-Rad), proteins were detected by immunoblotting with rabbit polyclonal antisera to Ste11, Ste7, Fus3, Ste4, and Ste5, as appropriate.
25. Qualitative mating tests were performed by patching the *MAT***a** strains to be tested on appropriate selective medium, and then replica-plating onto a lawn of DC17 on YP medium containing either 2% Gal/0.2% Suc or 2% Glc (depending on the promoter used for *STE5* expression) at 30°C overnight. The resulting mating plates then were replica-plated onto a minimal medium [synthetic complete (SC)] (*23*) selective for diploids and further incubated at 30°C overnight.
26. The GST fusions were generated as follows. A 640-bp fragment encoding *S. japonicum* GST was generated by PCR with a pGEX vector (Pharmacia) as the template and appropriate primers to install Ase I sites at both the 5′ and 3′ ends of the product, and inserted into the Nde I site in-frame and downstream of codon 913 of the *STE5* coding sequence in pCJ117 (*22*), creating a junction (5′-CATAATATGTCC-3′) encoding $His_{912}Asn_{913}$ Met-Ser (where Met is the first residue of GST), thus yielding pCJ148. pCJ149, expressing the Ste5(C177A C180A)-GST fusion, was created from pCJ119 in an analogous fashion. The translation stop codon in both pCJ148 and pCJ149 is provided by the natural TAG at the end of the *STE5* coding sequence.
27. *MAT***a** strains to be tested were grown in an appropriate selective medium (SC) (*23*) containing either 2% glucose (Glc) or 2% raffinose (Raf), depending on the promoter regulating *STE5* expression. Strains carrying plasmids expressing *STE5* constructs from the *GAL1* promoter were induced by addition of galactose (Gal) to a final concentration of 2% and incubation for 60 min before dilution and were plated on a medium containing 2% Gal and 0.2% sucrose (Suc). Samples (0.2 ml) of serial dilutions of the *MAT***a** strains were mixed, in triplicate, with 0.6 ml of a culture of a *MAT*α tester strain (DC17) that had been grown to midexponential phase in yeast extract–peptone (YP) (*23*) medium. Portions (0.4 ml) of these mixtures were plated on a medium lacking the appropriate supplements to select for diploids and incubated at 30°C for 36 to 40 hours. Corresponding dilutions of the *MAT***a** strains were also plated to determine the total number of viable haploids. Mating efficiencies were calculated as the ratio of diploid cells formed to the total number of input *MAT***a** haploids.
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Negative Regulation by HLA-DO of MHC Class II–Restricted Antigen Processing

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HLA-DM is a major histocompatibility complex (MHC) class II–like molecule that facilitates antigen processing by catalyzing the exchange of invariant chain–derived peptides (CLIP) from class II molecules for antigenic peptides. HLA-DO is a second class II–like molecule that physically associates with HLA-DM in B cells. HLA-DO was shown to block HLA-DM function. Purified HLA-DM-DO complexes could not promote peptide exchange in vitro. Expression of HLA-DO in a class II$^+$ and DM$^+$, DO$^-$ human T cell line caused the accumulation of class II–CLIP complexes, indicating that HLA-DO blocked DM function in vivo and suggesting that HLA-DO is an important modulator of class II–restricted antigen processing.

MHC class II molecules assemble in the endoplasmic reticulum (ER) as a nonameric complex consisting of an invariant chain trimer associated with three class II αβ dimers (*1*). Signals in the invariant chain cytoplasmic domain direct the complex into the endocytic pathway, where invariant chain degradation results in the transient formation of a class II αβ dimer with a residual fragment of the invariant chain, CLIP (class II–associated invariant chain peptides), in the peptide-binding groove (*2*). The interaction of the αβ-CLIP complex in the antigen-processing compartment, or MHC class II compartment (MIIC) (*3*), with a second class II–like molecule, called HLA-DM in humans and H-2M in mice, induces CLIP dissociation (*4*, *5*). Association of empty αβ dimers with DM stabilizes them until high-affinity peptides derived from internalized proteins can bind (*6*, *7*). Mature αβ-peptide complexes then leave the endocytic pathway and are expressed on the cell surface.

Another class II–like molecule, HLA-DO, expressed only in B cells and thymic epithelium (*8*–*12*), physically associates with DM in the ER and during and after transport to the MIIC (*8*). We therefore investigated what the effect of DO association might be on the ability of DM to catalyze CLIP dissociation and peptide loading. HLA-DM was affinity-purified (*4*) from the Burkitt's lymphoma B cell line Raji and from the DO-negative DM-transfectant T2/DM (*13*). Protein immunoblotting with rabbit antisera specific for the DM and DO-β chain cytoplasmic domains (*14*) showed that the purified material from Raji contained DO, whereas that from T2/DM did not (Fig. 1A). DM-DO association was maintained through extensive washing of the monoclonal antibody (mAb) MaP.DMB/c affinity column with sodium deoxycholate. Thus, the interaction is qualitatively different from that of DM with conventional class II molecules, which is retained only in mild detergents such as CHAPS or digitonin

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