Distinct functions of calmodulin are required for the uptake step of receptor-mediated endocytosis in yeast: the type I myosin Myo5p is one of the calmodulin targets

M.Isabel Geli, Andreas Wesp and Howard Riezman

Biozentrum of the University of Basel, CH-4056 Basel, Switzerland

1Corresponding author
e-mail: riezman@ubaclu.unibas.ch

The uptake step of receptor-mediated endocytosis in yeast is dependent on the calcium binding protein calmodulin (Cmd1p). In order to understand the role that Cmd1p plays, a search was carried out for possible targets among the genes required for the internalization process. Co-immunoprecipitation, two-hybrid and overlay assays demonstrated that Cmd1p interacts with Myo5p, a type I unconventional myosin. Analysis of the endocytic phenotype and the Cmd1p–Myo5p interaction in thermosensitive cmd1 mutants indicated that the Cmd1p–Myo5p interaction is required for endocytosis in vivo. However, the Cmd1p–Myo5p interaction requirement was partially overcome by deleting the calmodulin binding sites (IQ motifs) from Myo5p, suggesting that these motifs inhibit Myo5p function. Additionally, genetic and biochemical evidence obtained with a collection of cmd1 mutant alleles strongly suggests that Cmd1p plays an additional role in the internalization step of receptor-mediated endocytosis in yeast.

Keywords: calmodulin/endocytosis/Saccharomyces cerevisiae/type I myosin

Introduction

Endocytosis is the process whereby eukaryotic cells internalize extracellular material as well as part of their own plasma membrane, facilitating uptake of nutrients, down-regulation of receptors and removal of damaged proteins from the cell surface. The development of assays that quantitatively measure receptor-mediated internalization in the yeast Saccharomyces cerevisiae has facilitated identification of many genes specifically required in this process (Riezman et al., 1996 and references therein). Analysis of these genes indicates that the actin cytoskeleton plays a fundamental role in the endocytic uptake in yeast. (Kübler and Riezman, 1993; Raths et al., 1993; Benedetti et al., 1994; Kübler et al., 1994; Mumm et al., 1995; Geli and Riezman, 1996; Wendland et al., 1996; Wesp et al., 1997; Tang et al., 1997). Even though the requirement for the actin cytoskeleton in endocytic uptake in animal cells has been a matter of discussion for a long time, evidence now suggests that it also plays a role in both clathrin-dependent and -independent endocytic pathways (Lamaze et al., 1997).

The Ca²⁺ sensor, calmodulin is one of the proteins that may play a fundamental role in the regulation of the cytoskeleton re-arrangements required to form an endocytic vesicle. Calmodulin has been implicated in regulating actin–actin, actin–myosin and actin–plasma membrane associations in many cell types including yeast (reviewed in Clore et al., 1993; Jannney, 1994; Ohya and Botstein, 1994a,b; James et al., 1995). One single essential gene (CMD1) encodes calmodulin in Saccharomyces cerevisiae (CMD1) (Davis et al., 1986). Previous work from our laboratory has demonstrated that calmodulin plays a fundamental role in endocytosis (Kübler et al., 1994). A temperature sensitive (ts) calmodulin mutant (cmd1-1) was completely blocked for α-factor internalization almost immediately upon shift to the restrictive temperature. The calmodulin requirement is apparently calcium independent because the ts mutant, cmd1-3, impaired in Ca²⁺ binding in vitro (Geiser et al., 1991), allowed wild type (wt) internalization kinetics (Kübler et al., 1994). In addition, some evidence of a role for calmodulin in the uptake step of endocytosis in mammalian cells also exists. Calmodulin has been purified from clathrin coated vesicles and was shown to interact with clathrin light-chain (Salisbury et al., 1980; Pley et al., 1995). Further, endocytosis of the cell surface IgM receptor in lymphoblastoid cells is sensitive to the calmodulin directed drug, Stelazine (Salisbury et al., 1980).

In order to further understand the actin- and calmodulin-dependent mechanism that functions in endocytic internalization in yeast, we searched for calmodulin targets among the proteins known to be required for this process. Specifically, we investigated the physical and functional interaction between calmodulin and the unconventional type I myosin, Myo5p. Type I myosins are ubiquitous actin/ATP-dependent molecular motors that bear a short, positively-charged C-terminal domain that can bind acidic phospholipids and cellular membranes (reviewed in Korn and Hammer, 1990; Pollard et al., 1991; Tan et al., 1992; Mooseker and Cheney, 1995; Titus, 1997). Two functionally redundant genes encode for type I myosins in yeast: MYO3 and MYO5 (Goodson and Spudich, 1995; Géli and Riezman, 1996; Goodson et al., 1996). Myo5p seems to play a major role in endocytosis, because deletion of MYO5 (but not MYO3) caused a strong defect in α-factor internalization at elevated temperatures. Deletion of both genes caused a synthetically lethal phenotype that was complemented with a ts myo5 allele (myo5-1). The ts strain was completely blocked for internalization of α-factor and its receptor, Ste2p, at the restrictive temperature (Géli and Riezman, 1996). The role of type I myosin in the internalization step of endocytosis is not restricted to yeast. A strong defect in pinocytic uptake has also been described for Dictyostelium type I myosin mutants (Novak et al., 1995; Jung et al., 1996).

All myosins described thus far bear at least one IQ
consensus motif (IQXXXRXXXXR) at the neck between the motor head and the tail domains. The IQ motifs can constitute binding sites for small EF-hand-containing proteins, which are thought to work as calmodulin light-chains and may confer Ca\(^{2+}\) sensitivity to myosin regulation. Many vertebrate type I myosins co-purify with calmodulin and interestingly, maximal binding of calmodulin is generally achieved in the absence of Ca\(^{2+}\), in contrast to what is seen for many other calmodulin-activated enzymes (Wolenski, 1995).

Whether calmodulin is required for type I myosin function in vivo, and whether it regulates myosin activity in a Ca\(^{2+}\)-dependent manner, is still a matter of debate since this subject has mainly been addressed in vitro and the results obtained seem to vary depending on the myosin studied and the assay used to monitor its activity (Wolenski, 1995 and references therein). In addition, some type I myosins do not seem to bind calmodulin, and their Mg\(^{2+}\)-ATPase is fully active in the absence of light-chains (Maruta, 1978).

Here, we present evidence showing that calmodulin interacts with the type I myosin, Myo5p. Analysis of a collection of ts cmd1 mutants for their endocytic phenotype and their ability to bind Myo5p strongly indicates that Cmd1p–Myo5p interaction is required to drive endocytic internalization. This requirement is partially overcome by deletion of the calmodulin binding sites of Myo5p, suggesting that the IQ motifs may overlap with a Myo5p auto-inhibitory domain. In addition, we present genetic evidence suggesting that calmodulin also fulfills a Myo5p-independent function in the uptake step of receptor-mediated endocytosis in yeast.

**Results**

### Myo5p associates with Cmd1p

Since Myo5p and Cmd1p are both required for the uptake step of receptor-mediated endocytosis and interaction between calmodulin and type I myosins have been previously described in vertebrate systems, we decided to investigate whether Cmd1p and Myo5p physically interact and whether this interaction is relevant for their endocytic function in vivo.

To test for a physical interaction between Cmd1p and Myo5p, we raised a polyclonal serum against Cmd1p and we tagged Myo5p with a myc epitope for detection in co-immunoprecipitation experiments. The specificity of the α-Cmd1p antibody was confirmed by immunoblotting, comparing extracts from yeast expressing either wild type (wt) Cmd1p or a mutant calmodulin with slower SDS–PAGE mobility ( Cmd1-228p) (Figure 1A). Additionally, a difference in apparent molecular weight of the recognized band was observed in wt extracts depending on the presence of 100 mM EGTA or 100 mM CaCl\(_2\) in the SDS–PAGE loading buffer (Brockerhoff, 1992) (Figure 1A). The myc-tagged MYO5 was subcloned in a 2μ plasmid to create pA12mycMYO5 and transformed in a strain deleted for the genomic MYO5 (strain RH3976). As a control, the same strain was transformed with a plasmid bearing a non-tagged MYO5. Total protein extracts from each strain were separated by SDS–PAGE and immunoblotted using the α-myc monoclonal antibody (9E10). A band of the expected molecular weight (137 kDa) was detected exclusively in the extract from the strain expressing the myc-tagged Myo5p (Figure 1A). 2μ and centromeric plasmids bearing myc-tagged MYO5 fully complemented a myo5Δ strain by the following criteria: (i) they supported wt growth when combined with a MYO3 deletion; (ii) they supported α-factor uptake with wt kinetics in the presence or absence of Myo3p (data not shown). A native extract enriched for Myo5p was used for immunoprecipitation with the α-Cmd1p serum. Since maximal calmodulin binding has been observed in the absence of Ca\(^{2+}\) for most type I myosins, the whole procedure was performed in the presence of 1 mM EGTA.
The immunoprecipitates (P) and the supernatants (S) were then analyzed for Cmd1p or Myo5p content by immunoblot using the α-Cmd1p and the 9E10 antibodies, respectively (Figure 1B, EGTA). Myo5p was recovered in the Cmd1p immunoprecipitates under these conditions. The same results were obtained using α-Cmd1p serum or affinity purified α-Cmd1p IgG from another source [kindly provided by T.Davis, Washington, Seattle (data not shown)]. As controls, the same experiment was performed using a preimmune serum (Figure 1B, preim.) or a strain expressing the non-tagged version of Myo5p (Figure 1B, no myc). Similar to findings with other type I myosins, the Cmd1p–Myo5p interaction was disrupted with 5 mM CaCl2 in the immunoprecipitation buffer (Figure 1B, Ca2+). In the latter case, proteins were transferred to a nitrocellulose filter, partially renatured, and incubated with 35S-labeled purified recombinant Cmd1p. After washing, the filters were dried and exposed to an X-ray film. (D) Deletion of IQ1 and IQ2 disrupts Cmd1p–Myo5p interaction in a co-immunoprecipitation experiment. myo5A (strain RH3976) strains bearing pA12mycMYO5 (MYO5) or pA12mycmyo5-ΔIQ1ΔIQ2 (IQ1ΔIQ2) were grown to log-phase, harvested, lysed using glass beads and a high speed pellet was prepared. Myo5p was solubilized in a high salt buffer containing 5 mM ATP and 1 mM EGTA. Extracts were immunoprecipitated with the α-Cmd1p antibody. Immunoprecipitates (P) and supernatants (S) were processed for detection of Cmd1p or the myc-tagged wt or mutant Myo5p by immunoblotting using the α-Cmd1p or α-myc antibodies.

The immunoprecipitates (P) and the supernatants (S) were then analyzed for Cmd1p or Myo5p content by immunoblot using the α-Cmd1p and the 9E10 antibodies, respectively (Figure 1B, EGTA). Myo5p was recovered in the Cmd1p immunoprecipitates under these conditions. The same results were obtained using α-Cmd1p serum or affinity purified α-Cmd1p IgG from another source [kindly provided by T.Davis, Washington, Seattle (data not shown)]. As controls, the same experiment was performed using a preimmune serum (Figure 1B, preim.) or a strain expressing the non-tagged version of Myo5p (Figure 1B, no myc). Similar to findings with other type I myosins, the Cmd1p–Myo5p interaction was disrupted with 5 mM CaCl2 in the immunoprecipitation buffer (Figure 1B, Ca2+).

The IQ motifs in the neck region of Myo5p are necessary and sufficient for Cmd1p binding

Two IQ motifs are present at the Myo5p neck (IQ1 and IQ2) whose sequence and location in the protein is schematically represented in Figure 2A. In order to analyze whether any of these sequences could mediate binding to Cmd1p, we first used the two-hybrid system. We fused the neck region of Myo5p including the IQ motifs flanked by 20 amino acids N- and C-terminally, after the B42 transcriptional activator domain. CMD1 was fused to the lexA DNA binding domain. The constructs were transformed into EGY48 bearing the β-galactosidase reporter gene downstream of 8 LexA binding sites (pSH18–34) and interaction was monitored on X-Gal-containing plates (Figure 2B). A strong β-galactosidase activity was detected in the strain bearing the LexA–Cmd1p (CMD1) fusion and the B42-Myo5p neck region (IQ1IQ2) fusion protein. Deletion of either IQ1 (IQ1ΔIQ2) or IQ2 (IQ1ΔIQ2Δ) diminished, but did not abolish, interaction. Only deletion of both IQ motifs (IQ1ΔIQ2Δ) completely abrogated the interaction in this assay. As a control, LexA fused to the Drosophila melanogaster protein, bicoid, was used instead of the CMD1 fusion and it showed no interaction.

In order to confirm the specificity of the interaction between the Myo5p neck region and Cmd1p, we searched the yeast genome data bank for genes that encode proteins less than 200 amino acids in length with homology to calmodulin. Six were identified (DDBJ/EMBL/GenBank accession numbers M14078, Z72628, X69765, X74151, U25841 and U28373). Each gene was fused in-frame to the lexA binding domain coding sequence of pEG202. None of them interacted with the IQ1IQ2Δ2 prey construct, showing no detectable development of blue color (data not shown).

In order to confirm the specificity of the interaction between the Myo5p neck region and Cmd1p, we searched the yeast genome data bank for genes that encode proteins less than 200 amino acids in length with homology to calmodulin. Six were identified (DDBJ/EMBL/GenBank accession numbers M14078, Z72628, X69765, X74151, U25841 and U28373). Each gene was fused in-frame to the lexA binding domain coding sequence of pEG202. None of them interacted with the IQ1IQ2Δ2 prey construct, showing no detectable development of blue color (data not shown).

In order to confirm the specificity of the interaction between the Myo5p neck region and Cmd1p, we searched the yeast genome data bank for genes that encode proteins less than 200 amino acids in length with homology to calmodulin. Six were identified (DDBJ/EMBL/GenBank accession numbers M14078, Z72628, X69765, X74151, U25841 and U28373). Each gene was fused in-frame to the lexA binding domain coding sequence of pEG202. None of them interacted with the IQ1IQ2Δ2 prey construct, showing no detectable development of blue color (data not shown).

In order to confirm the specificity of the interaction between the Myo5p neck region and Cmd1p, we searched the yeast genome data bank for genes that encode proteins less than 200 amino acids in length with homology to calmodulin. Six were identified (DDBJ/EMBL/GenBank accession numbers M14078, Z72628, X69765, X74151, U25841 and U28373). Each gene was fused in-frame to the lexA binding domain coding sequence of pEG202. None of them interacted with the IQ1IQ2Δ2 prey construct, showing no detectable development of blue color (data not shown).

In order to confirm the specificity of the interaction between the Myo5p neck region and Cmd1p, we searched the yeast genome data bank for genes that encode proteins less than 200 amino acids in length with homology to calmodulin. Six were identified (DDBJ/EMBL/GenBank accession numbers M14078, Z72628, X69765, X74151, U25841 and U28373). Each gene was fused in-frame to the lexA binding domain coding sequence of pEG202. None of them interacted with the IQ1IQ2Δ2 prey construct, showing no detectable development of blue color (data not shown).

In order to confirm the specificity of the interaction between the Myo5p neck region and Cmd1p, we searched the yeast genome data bank for genes that encode proteins less than 200 amino acids in length with homology to calmodulin. Six were identified (DDBJ/EMBL/GenBank accession numbers M14078, Z72628, X69765, X74151, U25841 and U28373). Each gene was fused in-frame to the lexA binding domain coding sequence of pEG202. None of them interacted with the IQ1IQ2Δ2 prey construct, showing no detectable development of blue color (data not shown).
purified recombinant Cmd1p, dried and exposed to X-ray film. Consistent with the two-hybrid results, maximal binding to Cmd1p was observed for the IQ1IQ2 fusion protein (lane 1) and deletion of either IQ1 (lane 2) or IQ2 (lane 3) diminished, but did not abolish, interaction. No interaction was detected for any of the other constructs. These results indicated that the interaction between the Myo5p neck region and Cmd1p is direct.

In order to determine whether the IQ motifs are necessary for Cmd1p–Myo5p interaction in the context of the whole Myo5p molecule, a myc-tagged myo5 mutant was generated where the IQ motifs were deleted (myo5-IQ1IQ2Δ). This allele was subcloned into a 2µ plasmid and used for a co-immunoprecipitation experiment with the α-Cmd1p antibody as described above. Deletion of the IQ motifs completely abrogated Cmd1p–Myo5p interaction in this assay (Figure 2D).

**Deletion of the calmodulin binding sites in Myo5p only partially impairs its endocytic function**

In order to determine whether Cmd1p binding is required for Myo5p function, we substituted the mutant myo5-IQ1IQ2Δ for the wt MYO5 in the yeast genome. This strain was then crossed to a myo4Δ strain in order to generate the myo3Δmyo5-IQ1IQ2Δ double mutant. A representative tetrad dissection from the myo3ΔMYO3 myo5-IQ1IQ2Δ MYO5 diploid is shown in Figure 3A. As controls, myo3Δ MYO3 myo5ΔMYO5 and myo3ΔMYO3 MYO5/MYO5 strains were also dissected. As previously described, the predicted myo3Δ myo5Δ double mutant spores (myo3Δ MYO3 myo5ΔMYO5, filled circles) failed to grow in our strain background (Geli and Riezman, 1996). In contrast, myo3Δ myo5-IQ1IQ2Δ (myo3ΔMYO3 myo5-IQ1IQ2Δ MYO5, filled circles) showed wt viability and growth at 24°C, suggesting that Myo5-IQ1IQ2Δp was at least partially functional at this temperature. In order to determine whether the Myo5-IQ1IQ2Δp was functional for endocytosis, we assayed a myo3Δ myo5-IQ1IQ2Δ strain for α-factor uptake (Figure 3B, MYO5) and compared it with a myo3Δ MYO5 strain that shows wt internalization kinetics (Figure 3B, MYO5). Myo5-IQ1IQ2Δp was able to drive α-factor internalization in a myo3Δ mutant with a rate close to wt at 24°C. However, we observed that α-factor uptake and growth were impaired at higher temperatures. Uptake was completely blocked at 37°C (Figure 3B) and the inactivation was not reversible, suggesting that this protein might unfold at high temperatures. Mutant Myo5p proteins where only IQ1 or IQ2 were deleted sustained α-factor uptake kinetics with nearly wt kinetics, both at 24°C or 37°C (data not shown).

**Cmd1p fulfills at least two distinct functions in the uptake step of receptor-mediated endocytosis in yeast**

The data depicted above could indicate that Cmd1p binding to Myo5p is dispensable for the endocytic function and may suggest a role for Cmd1p as a negative regulator of myosin function. However, it could also be possible that Cmd1p binding to Myo5p is required to block an autoinhibitory domain of Myo5p which overlaps with the IQ motifs. In fact, the calmodulin binding sites of many calmodulin-activated enzymes have been mapped to amphiphilic α-helices that function as autoinhibitory domains (James et al., 1995). In this case, disruption of the Cmd1p–Myo5p interaction would only have an inhibitory effect on the uptake assay in the presence of the intact Myo5p, but not in the myo5-IQ1IQ2Δ strain.

In order to differentiate between these two hypotheses, we decided to disrupt the interaction between Cmd1p and wt Myo5p using cmd1 mutant alleles and to analyze these mutants in terms of their endocytic phenotype. Since calmodulin is a multifunctional protein, cmd1 alleles impaired in binding to distinct targets would greatly facilitate this approach. For that purpose, we decided to analyze the ts cmd1 mutant collection described by Ohya and Botstein (1994a, b). The ts recessive alleles generated by these authors can be classified into four intragenic complementation groups (A, B, C and D), each bearing distinct, major phenotypic defects: actin cytoskeleton disorganization, calmodulin delocalization, nuclear division and budding defects, respectively. These findings suggest that calmodulin fulfills at least four different essential functions in the yeast cell, which are specifically impaired in mutants belonging to the different intragenic complementation groups.

We assayed the different ts cmd1 alleles for their ability to internalize α-factor at the restrictive temperature (37°C). The results are summarized in Figure 4A. Cells were considered negative for endocytosis (−) if the α-factor uptake rates were less than 15% of the wt at 37°C. Cells were considered positive (+) if they showed near wt uptake kinetics. We observed that mutants from intragenic complementation groups A and B were negative for α-factor uptake at the restrictive temperature, whereas mutants from intragenic complementation groups C and D showed wt uptake kinetics. Consistent with this, alleles that could not complement mutants in groups B, C and D were also defective for α-factor uptake. We also found that one cmd1 allele, originally classified as partially dominant (cmd1-247) (Ohya and Botstein, 1994b), was defective in receptor-mediated internalization at the restrictive temperature.

The data presented above suggest that at least two distinct calmodulin functions are required for the uptake step of receptor-mediated endocytosis in yeast. Since the growth and endocytic defects are not necessarily caused by disruption of binding to the same Cmd1p target for a given cmd1 mutant, we redefined the intragenic complementation groups for the defective mutants (cmd1-226, cmd1-228, cmd1-247) in terms of their endocytic phenotype. For that purpose, we transformed the CMD1, cmd1-226, cmd1-228 and cmd1-247 strains with centromeric plasmids bearing the different alleles (pCMD1, pcmd1-226, pcmd1-228 and pcmd1-247) and assayed them for α-factor internalization at 37°C. The results, expressed as percentage of bound α-factor internalized per min in the linear range, are shown in Figure 4B. The results indicate that all the cmd1 mutants analyzed behave as recessive alleles in terms of their endocytic phenotype (including cmd1-247) because expression of wt Cmd1p completely restored uptake. This suggests that these mutations impair calmodulin function because it can no longer interact with the different targets. Furthermore, expression of Cmd1-228p and Cmd1-247p in the same cell restored α-factor internalization kinetics to wt levels suggesting that these two alleles affect two distinct functions, both required for
Myo5p is an endocytic target of calmodulin

Fig. 3. A mutant Myo5p lacking IQ1 and IQ2 (Myo5-IQ1Δ2Δp) is partially active. (A) myo3Δmyo5-IQ1Δ2Δ spores are viable. MYO5 or the mutant myo5-IQ1Δ2Δ alleles were integrated at the MYO5 locus in a myo5Δ strain (strain RH3978) and crossed to a myo3Δ strain (strain RH3977) to generate strains RH4013 and RH4014, respectively. Strains RH3375 (myo3ΔMYO3 myo5ΔMYO5), RH4013 (myo3ΔMYO3 MYO5/MYO5) and RH4014 (myo3ΔMYO3 myo5-IQ1Δ2Δ/MYO5) were sporulated and dissected. Filled circles indicate either predicted double mutants (for RH3375 or RH4014) or myo3ΔMYO5 spores (for RH4013) where the MYO5 allele is the integrated copy. (B) Myo5-IQ1Δ2Δp can partially sustain α-factor uptake in a myo3Δmyo5Δ background. A MATa myo3Δ myo5-IQ1Δ2Δ (strain RH3982, myo5-IQ1Δ2Δ) strain was assayed for its ability to internalize α-factor and compared with a myo3ΔMYO5 strain (strain RH3979, MYO5) that showed wt kinetics. Cells were grown to 0.5–1×10⁷ cells/ml, harvested and incubated in YPUATD on ice for 45 min in the presence of 35S-labeled α-factor. Internalization was triggered by resuspending the cells in 24°C or 37°C YPUATD. Samples were taken at the indicated time points. The percentage of total bound c.p.m. (pH 6 resistant) that were internalized (pH 1 resistant) are indicated. Values correspond to the mean of at least two independent experiments. Standard deviations were <15% of each value.

endocytosis. In contrast, the cmd1-226 allele was only partially complemented by cmd1-228 or cmd1-247, suggesting that this allele might affect both functions. We also observed that an extra copy of cmd1-247 partially restored endocytosis, suggesting that this mutant allele might be slightly weaker than cmd1-226 and cmd1-228 in terms of its endocytic defect. A partial suppression of the growth defect by expression of an extra copy of a cmd1 mutant allele has been described previously (Ohya and Botstein, 1994b).

Myo5p might be one of the Cmd1p targets for endocytosis

In order to analyze whether any of the cmd1 defective alleles were specifically affected in Myo5p function, we examined the effect of overexpressing MYO5 in the different cmd1 strains. If any of the cmd1 mutations resulted in a weakened interaction to Myo5p, the cause of the endocytic defect, overexpression of the target protein might restore α-factor uptake by increasing the amount of Myo5p–Cmd1p active complex per cell. We introduced a 2μ plasmid bearing MYO5 (p195mycMYO5) in the CMD1, cmd1-226, cmd1-228 and cmd1-247 strains. Overexpression of Myo5p in each strain was monitored by immunoblot using the EW and IK antibodies raised against peptides of the Myo5p tail (Figure 5, lower panel). Overexpression of Myo5p specifically restored α-factor internalization in the cmd1-247 mutant (Figure 5, upper panel) at the restrictive temperature (37°C).

The genetic interactions described above suggest the model depicted in Figure 5B for Cmd1p function in endocytosis. According to this model, Cmd1p interacts with two different targets that are both required for the uptake step of receptor-mediated endocytosis in yeast. Myo5p would be one of the targets. Cmd1p–Myo5p interaction would specifically be impaired in the cmd1-247 mutant, whereas the cmd1-228 mutation would affect binding to the other endocytic target. The cmd1-226 mutant allele would be affected in binding to both targets.

In order to test some of the predictions of this hypothesis, we examined the ability of the proteins encoded by the different cmd1 alleles to interact with Myo5p, using the two-hybrid system. The cmd1 mutant alleles belonging to complementation groups A, B, C or D were cloned after the lexA DNA binding domain coding sequence of pEG202 to assess their ability to interact with the neck region of
Fig. 4. At least two distinct Cmd1p functions are required in the uptake step of receptor-mediated endocytosis. (A) Analysis of the endocytic phenotype of the ts cmd1 mutant collection. cmd1 ts mutants classified into intragenic complementation groups A (strain RH3984), B (strain RH3985), C (strain RH3988 and RH3989), D (strain RH4015, RH4016 and RH4017) or B, C and D (strains RH4015, RH4016 and RH4017) or those classified as partially dominant (DOMINANT) (strain RH3990 and RH3991) (Ohya and Botstein, 1994a, b) were grown to 0.5–1×10^7 cells/ml, harvested and resuspended in 37°C YPUATD. After 10 min preincubation, 35S-labeled α-factor was added and samples were taken at 5, 10 and 15 min after addition of α-factor. (–) indicates uptake rates (percentage of c.p.m. internalized per min.) <15% of the wt rate. (+) indicates wt uptake kinetics. (B) Complementation analysis of the endocytic defect of cmd1-226, cmd1-228 and cmd1-247 mutants. CMD1 (strain RH3983), cmd1-226 (strain RH3984), cmd1-228 (strain RH3985) and cmd1-247 (strain RH3991) were transformed with the pRS416-derived centromeric plasmids bearing the different alleles (pCMD1, pcmd1-226, pcmd1-228, pcmd1-247). The cells were grown on SDYE-URA for plasmid selection and α-factor internalization monitored at 37°C as described above. The bars indicate percentage of α-factor counts internalized per min. Empty bars correspond to the strains bearing no plasmid. Black bars correspond to strains bearing the plasmid with the same allele as the genomic copy. Bars with identical patterns correspond to uptakes of cells expressing the same pair of alleles but with reciprocal genomic versus plasmid locations.

Myo5p. β-galactosidase activity was monitored on X-Gal containing plates as described above. As shown in Figure 6A, we observed that both cmd1-226 and cmd1-247 mutant proteins were strongly impaired in binding to the Myo5p neck region (IQ1IQ2) in agreement with the proposed model. All other cmd1 mutants, including cmd1-228, strongly activated transcription of the reporter gene. For some of the alleles (cmd1-228, cmd1-239 and cmd1-250), the specificity of the interaction could not be assessed on X-Gal-containing plates because they induced transcription of the reporter gene in the control strains bearing the Myo5p neck region where the IQ motifs were deleted (IQ1ΔIQ2Δ). Nevertheless, when liquid assays for β-galactosidase activity were performed on these strains, a significantly higher activity was detected in the strains bearing the IQ motifs (data not shown).

To confirm the differential behavior of Cmd1-226p and Cmd1-247p versus Cmd1-228p in terms of their ability to bind Myo5p, co-immunoprecipitation experiments using α-Cmd1p antibodies were performed. The different strains were transformed with a 2μ plasmid bearing the myc-tagged MYO5 (p195mycMYO5) and processed as described before. The results are shown in Figure 6B. As suggested by the two-hybrid experiments, Cmd1-226p and Cmd1-247p were impaired in Myo5p binding whereas the Myo5p–Cmd1-228p interaction was comparable with wt (Figure 6B).

Discussion

Myo5p interacts with Cmd1p through two IQ motifs in the Myo5p neck

The experiments presented above indicate that calmodulin and the unconventional myosin Myo5p interact in vivo and localize the Cmd1p binding site of Myo5p to the neck of this protein that includes two IQ motifs. The primary evidence that demonstrates the Cmd1p–Myo5p interaction is their co-immunoprecipitation from non-denatured extracts. The specificity of this interaction is strongly supported by the fact that co-immunoprecipitation was abrogated by defined mutations in either Myo5p or Cmd1p (myo5-IQ1Δ2Δ or cmd1-247 and cmd1-226). The essential role that both proteins play in the uptake step of receptor-mediated endocytosis and the suppression of the cmd1-247 endocytic defect by overexpression of MYO5 strongly suggest that this interaction occurs in vivo and is relevant to endocytosis.

The two-hybrid results, the overlay assay and the co-immunoprecipitation experiments define the Myo5p neck as sufficient, and the IQ motifs as necessary, for Cmd1p binding. Furthermore, the overlay assay shows that this interaction is direct. Both IQ motifs seem to contribute to this interaction since deletion of either one diminished, but did not abolish binding in the two-hybrid and the overlay assays. Using these techniques, we cannot deter-
targets. The target whose interaction with Cmd1p would be disrupted in the cmd1-226 mutant would be the unconventional type I myosin Myo5p. The neck is the major, if not the only Cmd1p binding site has mainly been addressed in vitro. This subject seems to be quite variable. Ca²⁺-dependent disruption of myosin–calmodulin interaction resulted in inhibition of an actin sliding assay using the avian Brush Border Myosin I (BBMI) and, therefore, a positive requirement of calmodulin for myosin function was suggested. However, Ca²⁺-dependent calmodulin release from the same myosin strongly activated its Mg²⁺-ATPase indicating a putative protein family (Maruta et al., 1978). This does not seem to be the case for Myo5p. A Cmd1p–Myo5p interaction was demonstrated using different techniques, whereas six different calmodulin-related proteins failed to interact with the Myo5p neck in the two-hybrid system. In this respect, the yeast type I myosins may resemble the vertebrate members of the group. Strikingly, the rat myosin I myr 3, which is also closely related to the protozoal type I myosins, has also been shown to use calmodulin as the light-chain (Wang et al., 1997).

**Myo5p–Cmd1p interaction is most likely required to support receptor-mediated internalization in vivo**

To date, the requirement of calmodulin for type I myosin function has never been demonstrated in vivo. This subject has mainly been addressed in vitro and the results obtained seem to be quite variable. Ca²⁺-dependent disruption of myosin–calmodulin interaction resulted in inhibition of an actin sliding assay using the avian Brush Border Myosin I (BBMI) and, therefore, a positive requirement of calmodulin for myosin function was suggested. However, Ca²⁺-dependent calmodulin release from the same myosin strongly activated its Mg²⁺-ATPase indicating a putative

---

**Fig. 5.** Overexpression of Myo5p specifically suppresses the uptake defect of cmd1-226. (A) CMD1 (strain RH3983), cmd1-226 (strain RH3984), cmd1-228 (strain RH3985) and cmd1-247 (strain RH3991) cells, were transformed with a 2μ plasmid bearing MYO5 (p195mycMYO5) (p195MYO5) or the empty plasmid (YEplac195) (p195) and the strains were assayed for α-factor uptake at the restrictive temperature (37°C) as described in Figure 4. The lower panel corresponds to the immunoblot analysis of the indicated strains using the EW and IK antibodies. (B) Model for Cmd1p endocytic function suggested by the genetic interactions. The cmd1-228 and cmd1-247 mutations would disrupt interaction with two different target proteins. Binding between Cmd1p and each of these targets would be required for the uptake step of receptor-mediated endocytosis in yeast. The target whose interaction with Cmd1p would be disrupted in the cmd1-247 mutant would be the unconventional type I myosin Myo5p. The cmd1-226 mutation would most likely affect binding to both targets.

**Fig. 6.** Cmd1p–Myo5p interaction is weakened by the cmd1-226 and the cmd1-247 mutations. (A) Two-hybrid analysis of the interaction between cmd1 alleles and the Myo5p neck. The indicated cmd1 alleles fused to the lexA DNA-binding domain of pJG202 were transformed into EGY48 bearing the β-galactosidase reporter gene (pSH18-34) and the Myo5p neck (pJG4-5IQ1IQ2) (IQ1IQ2) or the same sequence with the IQ motifs deleted (pJG4-5ΔIQ1ΔIQ2Δ) (IQ1ΔIQ2Δ) as control fused to the B42 transcriptional activator loop of pJG4-5. β-galactosidase activity was monitored on X-Gal plates as described in Figure 2B. (B) cmd1-226 and cmd1-247 mutations impair Myo5p–Cmd1p interaction in a co-immunoprecipitation experiment. CMD1 (strain RH3976) bearing pA12mycMYO5 or the cmd1-226 (strain RH3984), cmd1-228 (strain RH3985) and cmd1-247 (strain RH3991) mutants bearing p195mycMYO5 were grown to log-phase, harvested, lysed using glass-beads and a high speed pellet was prepared. myc-tagged Myo5p was solubilized in a high salt buffer containing 5 mM ATP and 1 mM EGTA. The extracts were immunoprecipitated using the α-Cmd1p antibody. The immunoprecipitates (P) and supernatants (S) were processed for immunoblot detection of Cmd1p and myc-tagged Myo5p using α-Cmd1p and α-myc antibodies, respectively.
role of calmodulin as a negative myosin regulator (Collins et al., 1990; Wolenski et al., 1993). The data presented in this article strongly suggest that Cmd1p–Myo5p interaction is essential for endocytosis. The fact that Cmd1p and Myo5p directly interact and that both proteins are required in the endocytic process already suggested this. However, more direct evidence comes from the study of the cmd1-247 mutant. The cmd1-247 mutation caused a recessive ts endocytic defect, as judged by the complementation experiments, suggesting that it impaired this process because Cmd1-247p could no longer interact with its relevant target for endocytosis. Consistent with these findings, we showed by two-hybrid and co-immunoprecipitation experiments that this mutation significantly weakened binding to Myo5p compared with the wt CMD1 or to other cmd1 mutations that are permissive for endocytic uptake. Additionally, overexpression of Myo5p specifically suppressed the endocytic defect of the cmd1-247 mutant, further indicating that Myo5p is the relevant Cmd1p target whose endocytic function is impaired in the cmd1-247 mutant and that this endocytic defect is caused by a lack of interaction between the two proteins. It should be mentioned that both the two-hybrid and the co-immunoprecipitation experiments revealed a weakened interaction between the Cmd1-226 and Cmd1-247 mutant proteins and Myo5p already at permissive temperature, suggesting that this type of analysis in vitro is more stringent than the conditions in vivo where a more short-lived interaction could suffice.

Since the Cmd1p–Myo5p interaction is required for endocytosis, it may appear surprising that deletion of the calmodulin binding site of Myo5p (myo3-ΔIQ1Δ2Δp) does not seem to have a major effect on myosin function at 24°C. Myo5-ΔIQ1Δ2Δp is at least partially functional for growth and endocytosis in a myo3Δmyo5-ΔIQ1Δ2Δ strain. A satisfactory explanation of this apparent discrepancy would be that the calmodulin binding sites overlap with an auto-inhibitory domain of Myo5p and that Cmd1p serves to counteract this inhibition. Indeed, many calmodulin-activated enzymes possess autoinhibitory domains that overlap with their calmodulin binding sites (James et al., 1995). Removal of the calmodulin binding sites from the cAMP phosphodiesterase (Klee et al., 1980), the plasma membrane Ca2+-pump ATPase (Zurini et al., 1984), the myosin light-chain kinase (Edelman et al., 1985) or the cysteine protease calpain (James et al., 1989) produced constitutively active enzymes, similar to what we observed for Myo5p in vivo. In addition, BBM1 head fragments, where the IQ motifs have been proteolytically removed, showed elevated Mg2+-ATPase activity, suggesting that the presence of an auto-inhibitory domain, which overlaps with the IQ motifs, may be a general feature of the type I myosins (Wolenski et al., 1993).

In addition, our results are in striking agreement with what has been described for the conventional myosin II regulatory light-chains (RLC). Removal of the RLC from the scallop myosin II using EDTA results in reduced ATPase and actin motility activity in vitro (Vale et al., 1984). In agreement, reduced amounts of RLC transcript in D.melanogaster produce phenotypes clearly associated to myosin II dysfunction (Karess et al., 1991; Warmke et al., 1992). In contrast, removal of the RLC binding site of the Dictyostelium myosin II renders a functional protein that is able to complement a strain deleted for the chromosomal myosin II gene and that shows even higher ATPase activity than the wt protein in vitro (Uyeda and Spudich, 1993).

The Myo5-ΔIQ1Δ2Δp does not seem to be functional at high temperatures. A myo3Δmyo5-ΔIQ1Δ2Δ strain showed impaired growth at 37°C and was completely blocked for α-factor uptake at this temperature. Two possible explanations for this can be proposed. First, calmodulin binding to Myo5p may only be required at elevated temperatures, even in the absence of the IQ motifs. Second, and perhaps more likely, the mutant protein may simply misfold or denature at high temperatures as a consequence of the deletion of the IQ region. Consistent with the latter explanation, Myo5-ΔIQ1Δ2Δp inactivation at 37°C was not reversible. On the other hand, the endocytic defect in the cmd1-247 mutant was fully reversible when cells were shifted back to permissive temperature, indicating that the cause of its endocytic defect at 37°C is different from that of the myo3Δ myo5-ΔIQ1Δ2Δ mutant.

**Cmd1p fulfills an additional function required in the uptake-step of receptor-mediated endocytosis**

The intragenic complementation of the cmd1-247 and cmd1-228 mutants in terms of their endocytic defect strongly indicates that Cmd1p fulfills at least two different functions, both required for the uptake step of receptor-mediated endocytosis in yeast. Expression of Cmd1-228p and Cmd1-247p proteins in the same cell restored α-factor uptake to wt levels, whereas strains expressing either mutant protein alone were completely blocked for receptor-mediated endocytosis at the restrictive temperature. This finding was not due to a dosage effect because it was allele specific and it was observed whether the cmd1-228 or the cmd1-247 allele were located on a centromeric plasmid or in the genome. The fact that both mutations are recessive for their endocytic phenotype, indicates that they most likely impair Cmd1p function by preventing Cmd1p binding to independent targets. Cmd1p–Myo5p binding seems to be much stronger in the cmd1-228 than in the cmd1-247 mutant as assessed by the two-hybrid and co-immunoprecipitation experiments, indicating that Myo5p is not the relevant Cmd1p target whose function is affected in cmd1-228. Consistent with this proposal, Myo5p overexpression did not rescue the cmd1-228 endocytic defect.

**Is Myo5p function regulated in a Ca2+-dependent manner?**

Data obtained in vitro with many different type I myosins suggest that calmodulin confers Ca2+-sensitivity to myosin regulation (Wolenski, 1995). For instance, the avian BBM1 copurified with four calmodulin molecules in the presence of EGTA. Addition of Ca2+ caused partial dissociation of calmodulin, concomitant with progressive inhibition of motility in an actin sliding assay (Collins et al., 1991; Wolenski et al., 1993). When the calmodulin binding sites of BBM1 were proteolytically removed, the resulting head fragment showed elevated Mg2+-ATPase activity, which was then completely insensitive to Ca2+ (Wolenski et al., 1993).

We observed that addition of CaCl2 disrupted Cmd1p–Myo5p interaction in a co-immunoprecipitation experi-
ment, suggesting that a $Ca^{2+}$-dependent regulation of Myo5p is potentially possible. However, whether Cmd1p–Myo5p dissociation occurs in response to micromolar variations in the intracellular $Ca^{2+}$ concentrations and whether a tight $Ca^{2+}$-regulated switch between activated and inactivated Myo5p states is essential to its function in vivo is still an open question. In fact, a $Ca^{2+}$-independent Cmd1p-binding requirement for Myo5p function in endocytosis is consistent with the endocytic phenotype of the cmd1-3 mutant allele. This mutant allele bears point mutations in the EF-hand motifs that strongly impairs its ability to bind $Ca^{2+}$ in vitro and causes a ts phenotype in vivo (Geiser et al., 1991). Nevertheless, this mutant is able to support receptor-mediated endocytosis at wt rates (Kübler et al., 1994). Consistently with this, we observed that the Cmd1-3p is able to bind Myo5p in the two-hybrid system (data not shown). Unfortunately, our $\alpha$- Cmd1p antibody does not recognize the Cmd1-3p mutant protein and therefore co-immunoprecipitation experiments to assess its $Ca^{2+}$-insensitivity with respect to Myo5p binding were not possible. If the Cmd1-3p is truly insensitive to $Ca^{2+}$ regulation in vivo, this would indicate that a tight $Ca^{2+}$-dependent modulation of Myo5p–Cmd1p interaction is not essential for its endocytic function. This view is supported by the fact that Myo5p-IQ1Δ2Δp can still sustain endocytosis, albeit less efficiently.

Nevertheless, a $Ca^{2+}$-dependent regulation of Myo5p could still be essential in other cellular processes where this protein might be involved (Geli and Riezman, 1996; Goodson et al., 1996). Additional experiments will be necessary to investigate the role of calcium in endocytosis and whether or not a tight calcium-dependent regulation of the active and the inactive states of Myo5p is required for its function in vivo.

In summary, the data depicted above show that Myo5p and Cmd1p physically interact in vivo and that this interaction is required for receptor-mediated endocytosis in yeast. The results are consistent with a role of Cmd1p as a Myo5p light-chain and suggest that Cmd1p functions by counteracting a Myo5p autoinhibitory domain. Furthermore, the results indicate that Cmd1p fulfills an additional function in the uptake step of receptor-mediated endocytosis. Current isolation and characterization of suppressors of the cmd1-228 endocytic defect may help to identify the second Cmd1p target required in the uptake step of receptor-mediated endocytosis in yeast.

Materials and methods

Yeasts, media, strains and genetic techniques

All yeasts used in this report are listed in Table 1 with their relevant genotypes. Unless otherwise mentioned, strains that did not bear plasmids were grown in complete media YP/2% glucose, 2% peptone, 1% yeast extract, 40 μg/ml uracil (Ura), 40 μg/ml adenine ( Ade) and 40 μg/ml tryptophan (Trp), 2% agar for solid media. Strains bearing plasmids were selected on SD minimal media (Dulic et al., 1991) except liquid cultures for co-immunoprecipitation or α-factor uptake assays, which were grown in SDYE (SD, 0.2% yeast extract). Sporulation, tetrad dissection and scoring of genetic markers was performed as described by Sherman et al. (1974). Recombinant lysate was purified from E.coli as described in Hicke et al. (1997). Transformation of yeast cells was accomplished by the lithium acetate method of Ito et al. (1983).

RH3975 to RH3982 were generated from RH3375 by inducing sporulation on minimal media, tetrad dissection and scoring of the adequate markers. RH3979 and RH3982 were generated as follows: RH3978 was transformed with pINMT05 or pINmyo5-IQ1Δ2ΔA (see below) cut with SmaI at the MYO5 5′ untranslated region in order to introduce the wild type (wt) at the MYO5 3′ UTR. This intro
duces a URA3 marker at the MYO5 locus, which can easily be followed in crosses. At least six different colonies that grew on SD-Ura plates from each transformation were then independently crossed to RH3977 to generate the RH4013 and RH4014 diploids that were selected on SD-Ura-histidine(His)-Trp. Diploids were subsequently sporulated, dissected and the tetrads scored for linkage between the URA3 and cmd1-3p markers to verify integration of MYO5 or myo5-IQ1Δ2ΔA at the MYO5 locus. M4Δa myo3Δ MYO5 or myo3myo5-IQ1Δ2ΔA segregants from each respective diploid were chosen and analyzed for expression of wt or mutant Myo5p by immunoblot using the EW and IK antibodies (see below). Segregants with similar expression levels were used to perform the biotinylation assays.

RH3983 to RH3991 and RH4015 to RH4017 were generated from the strains YOC200, YOC226, YOC228, YOC231, YOC233, YOC239, YOC250, YOC242, YOC247, YOC234, YOC235 and YOC251 (Ohya and Botstein, 1994b), respectively, by disrupting the BARI gene at its unique SsuI site using a bar1Δ::LYS2 disruption cassette (Kübler and Riezman, 1993).

DNA techniques and plasmid constructions

All DNA manipulations were performed according to standard techniques (Sambrook et al., 1989) unless otherwise specified. Restriction enzymes, Klenow and T4 DNA ligase were obtained from Boehringer Mannheim, New England Biolabs, United States Biochemical or Stratagene Cloning Systems. Plasmids were purified by the Qiagen plasmid purification kit (Qiagen), and transformation of E.coli was performed by electroporation (Dower et al., 1988). All PCRs for cloning purposes were performed with a DNA polymerase with proof reading activity (Pfu, Stratagene Cloning Systems) and a TRIO-thermoblock (Biometra GmBH). Oligonucleotides were synthesized by Microsynth GmBH. All constructs were sequenced using a United States Biochemical Sequenase II DNA sequencing kit and a sequi-gen DNA sequencing gel from Bio-Rad Laboratoires.

All plasmids used in this report are listed in Table II. myo5-IQ1Δ2Δa and myo5-IQ1Δ2ΔA alleles were generated by two step PCR from MYO5, deleting the sequences that encode IQRAWRFLQR (IQ1), IQKTRIREKG (IQ2) or both. All alleles were cloned at the SsuI site of the centromeric plasmid YCplac33 (Gietz and Sugino, 1988) where the EcoR1 to SmuI sites of the polylinker were previously deleted. Using this procedure, p33MYO5, p33myo5-IQ1Δ2Δ and p33myo5- IQ1Δ2ΔA were generated. The myc-tagged versions of MYO5 and myo5-IQ1Δ2ΔA were constructed on these plasmids by introducing a sequence encoding for 6xHis (EQKLISEEDL) (Evan et al., 1985) after the initiation codon (ATG) to create p33mycMYO5 and p33mycmyo5IQ1Δ2ΔA. The different alleles were subcloned into the BamHI and SphI sites of a 2µm version of YEp24 (Sotz and Linder, 1990), pASZ12 (kindly provided by Prof. P.Philippsen), to generate the pA12 series, or YEp1ac195 (Gietz and Sugino, 1988) to generate p195mycMYO5, pINMYO5 and pINmyo5- IQ1Δ2ΔA (used to integrat the alleles) were generated from p33MYO5 and p33myo5-IQ1Δ2ΔA, respectively, by deleting the ARS and CEN sequences using the SsuI and SpeI sites. The pRS416CMD1, pRS416cmd1-228, pRS416cmd1-228, and pRS416cmd1-247 were constructed by subcloning the Fla·BamHI fragment of each allele (from the original pRB616 series, Ohya and Botstein, 1994b) into pRS416 (Sikorski and Hieter, 1989). For pEG202-derived plasmids, the different cmd1 alleles were amplified by PCR from the ATG to the stop codon using the original pRB616 derived plasmids or genomic DNA as templates. Convenient restriction sites (BamHI, XhoI) were included in the primers in order to subclone the fragments in frame with the lexA DNA binding domain of pEG202 (Ruden et al., 1991). The pG4-S- derived plasmids were constructed as follows: primers with the appropriate restriction sites at the 5′ and 3′ ends (EcoRI and XhoI) were used to amplify by PCR the sequence encoding the neck region of Myo5p containing the IQ motifs flanked by 20 amino acids N- and C-terminal (pG4-SIQ1-2Q). The PCR fragments were subcloned in frame after the B42 acid loop of pG4-S. The same oligonucleotides were used to construct pG4-SIQ1 ΔQ2, pG4-SIQ1 ΔQ2 and pG4-SIQ1 ΔQ2 using p3myo5-IQ1Δ2Δa, p3myo5-IQ1Δ2Δa and p3myo5-IQ1Δ2ΔA respectively as templates. The pGE2X-7t series’ for expression in E.coli were constructed using exactly the same procedure, but using BamHI and EcoRI restriction sites to subclone the fragments in frame after the gene encoding for the glutathione S-transferase (GST) of pGE2X-2T (Pharmacia Biotech). pQEl1CMD1a was created by subcloning the CMD1 gene in frame after 643
the His, tag of pQE11 (Qiagen). CMD1 was amplified by PCR from genomic DNA and cloned into pQE11 (cut with BamHI blunt ended with Klenow, and cut with HindIII) using the Real site of CMD1, which cuts one codon upstream of the ATG, and an artificially introduced HindIII site downstream of the stop codon (after partial digestion).

**Protein techniques and antibodies**

SDS-PAGE for protein separation was performed as described (Laemmli et al., 1970) using a Minigel system (Hoefer Scientific Instruments). 15% acrylamide gels were used to separate Cmd1p and the GST-fusion proteins and 7.5% gels to separate Myo5p. High and low range SDS–PAGE molecular weight standards (Bio-Rad Laboratories) were used for determination of apparent molecular weight. Total yeast protein extractions were performed as described (Horvath and Riezman, 1994), except that 3 x 10^7 cells were collected (at a culture density of 1.5 x 10^7 cells/ml) and frozen at –20°C and thawed before extraction. Western blotting was also performed as described (Horvath and Riezman, 1994) with the addition of 0.1% (v/v) Nonidet P-40 in the solutions for blocking.

### Table I. Yeast strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH3375</td>
<td>Δαde2AD2 his3his5 myo3α::HIS/MY05 myo5Δ::TRP1/MY05 leu2/leu2 lys2/lys2 trp1/trp1</td>
<td>Geli and Riezman (1996)</td>
</tr>
<tr>
<td>RH4013</td>
<td>Δhis5his3 myo3α::HIS/MY03 MY05::URA3::myo5Δ::TRP1/MY05 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 bar1/bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH4014</td>
<td>Δhis5his3 myo3α::HIS/MY03 MY05::URA3::myo5Δ::TRP1/MY05 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 bar1/bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3975</td>
<td>Δade2 his3 leu2 trp1 ura3 bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3976</td>
<td>Δade2 his3 myo5α::TRP1 leu2 trp1 ura3 bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3977</td>
<td>Δhis5 his3 myo3α::HIS/MY02 trp1 ura3 bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3978</td>
<td>Δhis5 myo3α::HIS/MY02 TRP1 leu2 lys2 trp1 ura3 bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3979</td>
<td>Δhis5 his3 myo3α::HIS/MY05::URA3::myo5Δ::TRP1 leu2 lys2 trp1 ura3 bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3982</td>
<td>Δhis5 myo3α::HIS/MY05::URA3::myo5Δ::TRP1 leu2 lys2 trp1 ura3 bar1</td>
<td>this study</td>
</tr>
<tr>
<td>RH3983</td>
<td>Δade2 ade3α::CMD1::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3984</td>
<td>Δade2 ade3α::cmd1-226::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3985</td>
<td>Δade2 ade3α::cmd1-228::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3986</td>
<td>Δade2 ade3α::cmd1-231::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3987</td>
<td>Δade2 ade3α::cmd1-233::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3988</td>
<td>Δade2 ade3α::cmd1-239::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3989</td>
<td>Δade2 ade3α::cmd1-250::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3990</td>
<td>Δade2 ade3α::cmd1-242::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH3991</td>
<td>Δade2 ade3α::cmd1-247::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH4015</td>
<td>Δade2 ade3α::cmd1-234::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH4016</td>
<td>Δade2 ade3α::cmd1-235::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>RH4017</td>
<td>Δade2 ade3α::cmd1-251::HIS3 cmd1Δ::TRP1 his3 leu2 lys2 trp1 ura3 bar1Δ::LYS2</td>
<td>this study</td>
</tr>
<tr>
<td>EGY48</td>
<td>Δura3 trp1 his3 leu2::lexAop6-LEU2</td>
<td>Gyuris et al. (1993)</td>
</tr>
</tbody>
</table>

### Table II. Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Yeast Ori</th>
<th>Yeast Marker</th>
<th>Insert</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeplac195</td>
<td>2μ</td>
<td>URA3</td>
<td>–</td>
<td>Gietz and Sugino (1988)</td>
</tr>
<tr>
<td>Ycplac33</td>
<td>CEN</td>
<td>URA3</td>
<td>–</td>
<td>Gietz and Sugino (1988)</td>
</tr>
<tr>
<td>p195mycMY05</td>
<td>2μ</td>
<td>URA3</td>
<td>mcy-tagged MY05</td>
<td>this study</td>
</tr>
<tr>
<td>pA12MY05</td>
<td>2μ</td>
<td>ADE2</td>
<td>MY05</td>
<td>this study</td>
</tr>
<tr>
<td>pA12mycMY05</td>
<td>2μ</td>
<td>ADE2</td>
<td>mcy-tagged MY05</td>
<td>this study</td>
</tr>
<tr>
<td>pA12mycMyo5-IQ1Δ2Δ</td>
<td>2μ</td>
<td>ADE2</td>
<td>mcy-tagged myo5-IQ1Δ2Δ</td>
<td>this study</td>
</tr>
<tr>
<td>pLexMY05</td>
<td>URA3</td>
<td>URA3</td>
<td>MY05</td>
<td>this study</td>
</tr>
<tr>
<td>pLexMY05-IQ1Δ2Δ</td>
<td>URA3</td>
<td>URA3</td>
<td>myo5-IQ1Δ2Δ</td>
<td>this study</td>
</tr>
<tr>
<td>pA16cmd1-226</td>
<td>CEN</td>
<td>CMD1</td>
<td>cmd1-226</td>
<td>this study</td>
</tr>
<tr>
<td>pA16cmd1-228</td>
<td>CEN</td>
<td>CMD1</td>
<td>cmd1-228</td>
<td>this study</td>
</tr>
<tr>
<td>pA16cmd1-247</td>
<td>CEN</td>
<td>CMD1</td>
<td>cmd1-247</td>
<td>this study</td>
</tr>
<tr>
<td>pG4-SIQ1Δ2Q</td>
<td>2μ</td>
<td>TRP1</td>
<td>B42IQ1Δ2Q</td>
<td>this study</td>
</tr>
<tr>
<td>pG4-SIQ1Δ2Q</td>
<td>2μ</td>
<td>TRP1</td>
<td>B42IQ1Δ2Q</td>
<td>this study</td>
</tr>
<tr>
<td>pG4-SIQ1Δ2Q</td>
<td>2μ</td>
<td>TRP1</td>
<td>B42IQ1Δ2Q</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202CMD1</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-226</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-226</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-228</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-228</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-231</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-231</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-233</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-233</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-239</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-239</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-250</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-250</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-242</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-242</td>
<td>this study</td>
</tr>
<tr>
<td>pEG202cmd1-247</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexACMD1-247</td>
<td>this study</td>
</tr>
<tr>
<td>pSH18-34</td>
<td>2μ</td>
<td>URA3</td>
<td>8 lexA Op. lacZ</td>
<td>Gyuris et al. (1993)</td>
</tr>
<tr>
<td>prFHIM-1</td>
<td>2μ</td>
<td>HIS3</td>
<td>lexAbicoid</td>
<td>Gyuris et al. (1993)</td>
</tr>
<tr>
<td>pGEX-CT</td>
<td>–</td>
<td>–</td>
<td>GST</td>
<td>this study</td>
</tr>
<tr>
<td>pGEX-2TQ1Δ2Q</td>
<td>–</td>
<td>–</td>
<td>GST/Q1Δ2Q</td>
<td>this study</td>
</tr>
</tbody>
</table>
and subsequent incubations. Incubation with primary antibodies, 9E10, α-Cmd1p, EW or IK (see below), was followed by incubation with the secondary antibody conjugated to peroxidase. Membranes were developed using ECL chemiluminescent detection reagent kit (Amersham Life Science), exposed to Kodak XAR X-ray films and developed using a Fuji automatic film developer.

The antibody against Cmd1p (α-Cmd1p) was raised in rabbits by injecting purified Cmd1p (see below). Two different antibodies were raised in rabbits against the Myo5p peptides EANEDDEDDED (EW) and ECPPQQNASIK (IK) synthesized by Neoseystem Laboratories (Strasbourg, France). Myo5p antibody specificity was assessed on immunoblots by comparing MYO5 and myo5ΔA extracts. The 9E10 anti-myc antibody was kindly provided by P.Ramage (Novartis AG, Basel, Switzerland).

**Extraction of Myo5p and co-immunoprecipitation with Cmd1p**

Extraction of Myo5p and co-immunoprecipitation experiments were performed based on Swanljung-Collins and Collins (1991) and Brockerhoff et al. (1994). Cells bearing the plasmids with myc-tagged wt or mutant MYO5 were grown to a density of 2x10^7 cells/ml. 3x10^8 cells were used for each immunoprecipitation. Cells were harvested in PAGE sample buffer (Supernatant). One-tenth of each sample was washed once with cold acetone and resuspended in 30 μl of buffer A (10 mM Imidazole–CI, 4 mM EDTA, 1 mM EGTA, 0.02% NaN3, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin pH 7.3) on ice. Cells were lysed using glass beads. Unbroken cells and debris were eliminated by centrifuging at 2500 g at 4°C for 5 min and the supernatants were transferred to 1.5 ml polyallomer microfuge tubes (Beckman Instruments). Further purification steps were carried out at 4°C. Samples were centrifuged at 100 000 g for 15 min, in a TL100 Beckman table top ultra-centrifuge in a TLA-100 rotor. The supernatant was discarded and the pellet washed again in 300 μl of buffer A. After a second centrifugation, the pellet was extracted twice with 100 μl of buffer B (100 mM Imidazole–CI, 0.2 M KCl, 5 mM MgCl2, 5 mM ATP, 1 mM EGTA, 0.02% NaN3, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin pH 6.8) for solubilization of Myo5p by first resuspending the samples in buffer B and then centrifuging for 20 min at 100 000 g. Both supernatants were recovered and transferred to an Eppendorf tube. Each sample (200 μl) was incubated on ice with slow rocking, with α-Cmd1p or preimmune serum previously bound to protein A sepharose. For the immunoprecipitations in the presence of Ca^2+ (CaCl2), the final concentration of 1 mM. A denaturing procedure was used to purify Cmd1p for antibody injection. 500 ml culture yielded ~7.5 mg of Cmd1p >95% pure as judged by SDS–PAGE and staining. 35S-labeled Cmd1p was purified from E.coli expressing Cmd1p (Brockerhoff et al. 1992) after labeling an IPTG-induced culture (OD600 of 0.25–0.3) with 10 μCi of 35SO42– (Amersham Life Science). The peak fraction contained M Cmd1p greater than 95% pure as judged by SDS–PAGE and staining.35S-labeled Cmd1p was cotransformed with the appropriate pEG202- and pG4-5-derived plasmids and streaked out on X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside) containing SD/Gal/Raf/His-Trp-Ura plates (0.67% yeast nitrogen base (Difco), 7 g/l NaHPO4, 3 g/l Na2HPO4, 2% galactose, 1% raffinose, 40 mg/ml leucine, 80 mg/l X-Gal (Sigma Chemicals Co.), 2% agar pH 7). Pictures were taken after 3 days of growth at 24°C. At temperatures >30°C, all lexA-calmodulin fusions gave unspecific lacZ transcription. Expression of all the lexA–calmodulin fusion proteins was confirmed by immunoblotting using α-Cmd1p antibodies. pEG202/ cmd1-228, pEG202/cmd1-239 and pEG202/cmd1-250 unspecifically activated lacZ transcription already at 24°C. However, when liquid assays for β-galactosidase activity were performed according to Miller (1972), a significant higher activity was detected in the corresponding samples bearing the pEG45-4IQ1H2Q plasmid than in those bearing the pEG45-4IQ1M23A plasmid (not shown).

**α-factor uptake assay**

[35S]α-factor uptake assays were performed as described by Dulie et al. (1991). For the MYO5 strains, a pulse and chase protocol was used. For the ts calmodulin mutants, a continuous presence assay was used. Cells were incubated at 37°C for 10 min before addition of α-factor. The samples were processed as described above. The internalization rates were calculated as the percentage of counts internalized per min between 5 and 10 min (linear range). All uptake assays were performed at least twice and the mean and standard deviations calculated for each time point.

**Acknowledgements**

We thank members of the Riezman laboratory for discussion and K.D’Hondt, A.Alconada and S.K.Lennon for critical reading of the manuscript. We are grateful to D.Botstein for sending the cmd1 strains and plasmids, to R.Brent for sending the two-hybrid strains and plasmids and to T.N.Davis for sending Ε-Cmd1p antibodies. We acknowledge the technical assistance of N.Benedetti, T.Derle, and T.Aust. This work was funded by the Canton Basel-Stadt and by a grant to H.R. from the Swiss National Science Foundation. M.I.G. was a recipient of an EMBO fellowship.

**References**


Myo5p is an endocytic target of calmodulin.


Received September 30, 1997; revised November 18, 1997; accepted November 26, 1997