Dominant-negative alleles of 14-3-3 proteins cause defects in actin organization and vesicle targeting in the yeast *Saccharomyces cerevisiae*

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Abstract 14-3-3 Proteins are thought to function as adapters in signaling complexes [1,2], thereby participating in cellular processes including vesicle trafficking and exocytosis [3,4]. To delineate further the function of 14-3-3 proteins during vesicle trafficking, we generated dominant-negative alleles of the two 14-3-3 homologues, Bmh1p and Bmh2p, in budding yeast and analyzed their phenotype in respect to exocytosis. Cells overexpressing the carboxy-terminal region of Bmh2p failed to polarize vesicular transport although bulk exocytosis remained unaffected and showed a disrupted actin cytoskeleton. Our data suggest that 14-3-3 proteins may act primarily on the actin cytoskeleton to regulate vesicle targeting.

Key words: Vesicle trafficking; Exocytosis; Actin cytoskeleton

1. Introduction

14-3-3 Proteins represent a family of ubiquitously expressed dimeric proteins of approximately 30 kDa. Homologues of this family are present in all eukaryotic cells and several isoforms are known [1,5]. These proteins are implicated in pleiotropic functions ranging from activation of tyrosine and tryptophan hydroxylases [6], regulation of protein kinase C [7–10], cell cycle regulation [11] to exocytosis [3,4]. In addition, they were identified as binding partners for many signaling proteins including Raf 1 kinase [11–14], Bcr-Abl [15] and PI3 kinase [16]. It is believed that the variety of binding partners and the participation in numerous cellular processes is due to their role as adapter proteins, which are involved in the spatial organization of signaling complexes [1,2].

14-3-3 Proteins have been shown to modulate vesicle trafficking and exocytosis in different experimental systems, although little is known about the signaling cascades involved here. In permeabilized adrenal chromaffin cells, 14-3-3 proteins were found to reconstitute catecholamine secretion [3,17], which was accompanied by a rapid and transient disassembly of the actin network [18]. This is likely to facilitate the movement of secretory granules towards the plasma membrane prior to granule docking and fusion [18]. In *Drosophila*, a 14-3-3 zeta homologue termed *LEONARDO* was discovered in a screen for learning and memory mutants [4,19]. Mutations in *LEONARDO* caused a defect in synaptic transmission which became more severe upon high frequency stimulation, when more vesicle turn-over in the synapse was required [20]. In a more general aspect of vesicle transport, two 14-3-3 homologues in the yeast *Saccharomyces cerevisiae*, Bmh1p and Bmh2p, were identified in a screen for gene products whose overexpression compensates an endocytosis defect caused by clathrin heavy chain deletion [21].

In order to further delineate the function of 14-3-3 protein in vesicle transport, we turned to the budding yeast *S. cerevisiae*, a well-established model system for polarized vesicle trafficking with the additional advantage that it possesses only two 14-3-3 genes, *BMH1* and *BMH2*, in contrast to higher eukaryotes. Deletion of single *BMH* genes does not affect cell viability [21,22], but double deletions of *BMH1* and *BMH2* are generally lethal [21,22].

The yeast 14-3-3 Bmh1p and Bmh2p proteins share 92% amino acid identity with each other and approximately 60% with 14-3-3 orthologues from higher eukaryotes. Here, we exploited this high extent of sequence conservation between yeast and mammalian isoforms to design a dominant-negative approach in yeast by overexpressing individual domains of the yeast 14-3-3 proteins. A phenotypic analysis of yeast cells overexpressing the carboxy-terminal domain of Bmh2p revealed that these cells are increased in cell size, they no longer polarize post-Golgi secretory transport and their actin cytoskeleton is disrupted. We conclude that the yeast 14-3-3 proteins are involved in pathways that control the organization of the actin cytoskeleton and thereby modulate vesicle targeting and cell polarity.

2. Materials and methods

2.1. Yeast genetic techniques

Cultures were grown in rich medium (YP) containing 1% (w/v) Bacto yeast extract and 2% (w/v) Bacto peptone (Difco) or in synthetic medium containing 0.7% (w/v) yeast nitrogen base without amino acids (Difco). Synthetic medium was supplemented with nutrients if necessary as described [25]. Glucose was generally used as a carbon source (2% (w/v) final concentration) except in experiments requiring expression from the *GAL1* promoter. In this case, cells were pregrown in 2% (w/v) raffinose to the early log phase and then, expression from the *GAL1* promoter was turned on by addition of 2% (w/v) galactose to the medium. Yeast transformations were performed with the lithium-acetate method [24] and transformants were selected on synthetic media supplemented with nutrients as required.

2.2. Generation of 14-3-3 domains for expression in yeast

Yeast 14-3-3 (*BMH1* and *BMH2*) fragments (see Fig. 1) were generated by PCR with *Bam*HI (at the 5′ end) and *Hin*dIII or *Bam*HI (at the 3′ end) overhangs. For internal fragments, start and stop codons were added. The PCR fragments were subcloned into a yeast integrating vector containing a *GAL1* promoter and *ADH* terminator with *URA3* as the auxotrophic marker (kindly provided by Peter Novick). All inserts were verified by DNA sequencing. The resulting plasmids were linearized with *Stu*I to allow for homologous recombination and integration at the site of the auxotrophic marker *URA3*.

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2.3. Immunofluorescence

Immunofluorescence was carried out as described before [26] with modifications [27]. Yeasts were grown at 25°C to an OD_{600} of approximately 0.2 in YP containing 2% (v/v) glycerol. Overexpression of proteins from the *GAL1* promoter was induced by addition of galactose (2% (w/v) final concentration) directly to the medium. Cells were grown for a further 10 h before fixation. Temperature-sensitive mutants were grown in YP containing 2% (w/v) glucose at 25°C to the mid-log phase and then shifted for 1 h to 37°C before harvesting and fixing. Anti-Sec4p antibody (provided by P. Novick) was used at 1/1000 dilution for 1 h at room temperature. Secondary antibody incubation was for 30 min at room temperature with Cy3-conjugated goat anti-rabbit antibodies at 1/500 dilutions. Cells were observed with a Zeiss Axiophot using a 100× objective unless otherwise stated and photographed using TMAX 400 film (Kodak).

2.4. Actin and 4′,6′-diamidino-2,4-phenylindole (DAPI) staining

Actin and DAPI stainings were essentially performed as described previously [28]. Cells were grown as before for 10 h to induce expression from the *GAL1* promoter. Cells were observed with a Zeiss Axiophot using a 100× objective. Pictures were taken and assembled as described above.

2.5. Invertase secretion

Invertase secretion was performed as described [29] with some modifications. All yeast strains were pre-grown in galactose as above to the mid-log phase. To measure invertase secretion from cells grown in galactose, the expression of invertase had to be repressed before the start of the experiment by shifting the cells to 2% (w/v) glucose for 1 h. This treatment did not affect the morphology of the cells. Further processing of the cells for determining invertase activity was as described [29].

Liberated glucose generated by invertase activity was measured in microtiter plates using a colorimetric assay [30]. Fifteen μl of each sample was incubated for 30 min at 37°C with 100 μl assay mix II (500 U glucose oxidase, 2.5 μg/ml peroxidase, 0.15 μg/ml *o*-dianisidine, 20 μM *N*-ethyl maleimide in phosphate-buffered saline). Then, the reaction was terminated by addition of 100 μl 6 N HCl. The absorbance of the samples was read at A_{540} in an enzyme-linked immunosorbent assay reader and the percentage of invertase secreted from the total cellular invertase content during the 1 h time interval was calculated. The total cellular invertase contents were normalized to 100%. The experiment was performed three times and samples were analyzed in duplicates each time.

3. Results

Previous data on mammalian 14-3-3 proteins had shown that the amino-terminal region is required for homo- and hetero-dimerization, while the carboxy-termini may determine their specificity for binding to distinct partner proteins [31–33]. To identify regions in the yeast 14-3-3 homologues, Bmh1p and Bmh2p, that are functionally important in vivo, we generated fragments encompassing various domains of Bmh1p and Bmh2p. These fragments were then conditionally overexpressed in yeast in an attempt to competitively interfere with the function of the endogenous Bmh proteins.

The following fragments depicted in Fig. 1A were tested: (i) the amino-termini (NT/BMH1 and NT/BMH2), i.e. regions corresponding to the dimerization interface in the mammalian 14-3-3 isoforms, (ii) fragments of the extreme carboxy-terminal domains (CT/BMH1 and CT/BMH2), which are analogous to the putative effector protein binding site in the mammalian 14-3-3 proteins, and (iii) a fragment of the putative partner protein binding groove that is identical in the two yeast 14-3-3 homologues, termed H56/BMH1/2. Effects of the induced Bmh1p and Bmh2p domains were then scored by comparing the growth of the stable integrants on media containing galactose where expression from the *GAL1* promoter is induced to growth on media containing glucose, where the *GAL1* promoter is repressed.

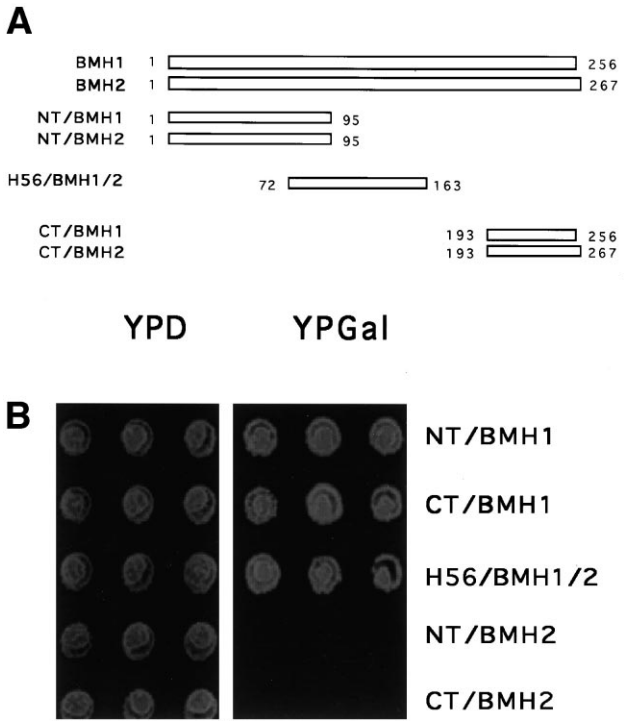

Fig. 1. Effects of overexpression of Bmh1p and Bmh2p fragments on cell growth. (A) Schematic representation of the deletion constructs generated. The upper panel indicates the sizes of the full-length open reading frames for Bmh1p and Bmh2p. The lower panel depicts the various deletion mutants generated. NT/BMH1 and NT/BMH2: amino-terminus of Bmh1p and Bmh2p, respectively. CT/BMH1 and CT/BMH2: carboxy-terminus of Bmh1p and Bmh2p, respectively. H56/BMH1/2: domain common to both Bmh1p and Bmh2p. Numbers next to the cartoons indicate amino acid numbers. (B) Cell growth after overexpression of deletion mutants of Bmh1p and Bmh2p. Three independent clones containing deletion constructs of either Bmh1p or Bmh2p under the control of the inducible *GAL1* promoter were grown at 25°C on rich medium in the presence of glucose (YPD) or galactose (YPGal). In the presence of glucose, the expression from the *GAL1* promoter was repressed, while in the presence of galactose, expression from the *GAL1* promoter was induced. Yeast strains abbreviated according to their respective integrated construct are depicted on the right. Note that only strains carrying alleles of NT/BMH2 and CT/BMH2 act dominant-negative upon growth on galactose.

Fig. 1B shows that some of the transformants grew on glucose and not on galactose, as predicted for dominant-negative alleles. This pattern of growth was temperature-independent since the same pattern was observed at 25, 30 and 37°C (data not shown). In this overexpression assay, the amino- and carboxy-terminal domains of Bmh2p, but not of Bmh1p, were dominant-negative for growth, whereas the shared domain H56/BMH1/2 was not. Interestingly, the dominant-negative regions of Bmh2p correspond to those regions of mammalian 14-3-3 orthologues that are supposed to be involved in dimer formation (NT/BMH2) or effector protein binding (CT/BMH2) [31–33].

To understand the function of the yeast 14-3-3 proteins, we analyzed the phenotypes of cells expressing dominant-negative alleles of Bmh2p, NT/BMH2 and CT/BMH2. Light microscopy showed that the overall sizes of these cells were approximately 40–50% larger than wild-type cells (data not shown).

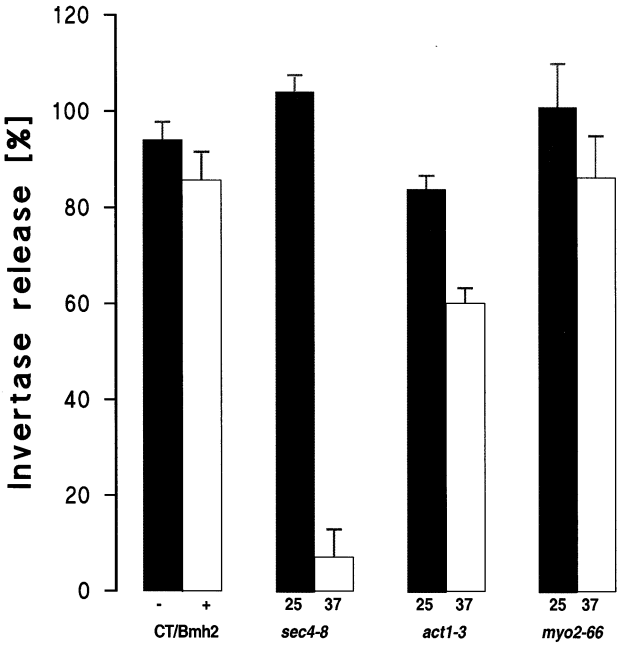

Fig. 2. Invertase secretion is not altered upon overexpression of the carboxy-terminus of Bmh2p. Invertase secretion from wild-type parental cells (−CT/BMH2), cells conditionally overexpressing the carboxy-terminus of Bmh2p (+CT/BMH2) and cells with temperature-sensitive mutations in Sec4p (*sec4-8*), actin (*act1-3*) or the unconventional myosin Myo2p (*myo2-66*) at the permissive (25°C, '25') or the restrictive (37°C, '37') temperature were measured as described under Section 2. The amount of secretion during a 1 h time interval was calculated. The experiment was performed in duplicates three times. The error bars indicate the S.E.M. ($n = 6$).

No apparent phenotypic differences were seen between cells expressing the amino- or the carboxy-terminal regions of Bmh2p. Therefore, all subsequent experiments were performed only with cells overexpressing the carboxy-terminus of Bmh2p, CT/BMH2.

As 14-3-3 proteins have been implicated in the modulation of exocytosis in both mammalian cells [3] and *Drosophila* [19], we examined whether the extent of secretion was altered in yeast expressing CT/BMH2. To this end, we measured constitutive secretion of invertase from post-Golgi vesicles into the periplasmic space by a colorimetric assay [29,30]. Fig. 2 shows that the percentage of invertase secretion in cells with dominant-negative 14-3-3 alleles was only marginally reduced. As a control, invertase secretion from a temperature-sensitive secretion mutant, *sec4-8*, was measured in parallel. Here, invertase secretion was reduced by approximately 90% when the cells were shifted to the restrictive temperature (Fig. 2) [29]. We conclude from these data that 14-3-3 proteins do not participate in the final stages of vesicle docking and fusion.

Measuring invertase secretion only mirrors bulk exocytosis and potential defects in vesicle targeting are not detected. Indeed, invertase secretion was not drastically reduced in the two cytoskeleton mutants, *act1-3* and *myo2-66*, which were also analyzed here for their ability to secrete invertase at the permissive and restrictive temperatures (Fig. 2). These mutants are known to exhibit vesicle trafficking defects [34]. In both *act1-3* and *myo2-66* strains, vesicles are no longer targeted to the bud tip and the neck region [26,35]. As overexpression of CT/BMH2 causes a morphology similar to that of *act1-3* and *myo2-66* mutants [34,35], we considered that the polarized transport of vesicles to the bud tip or the neck region may be defective after CT/BMH2 overexpression.

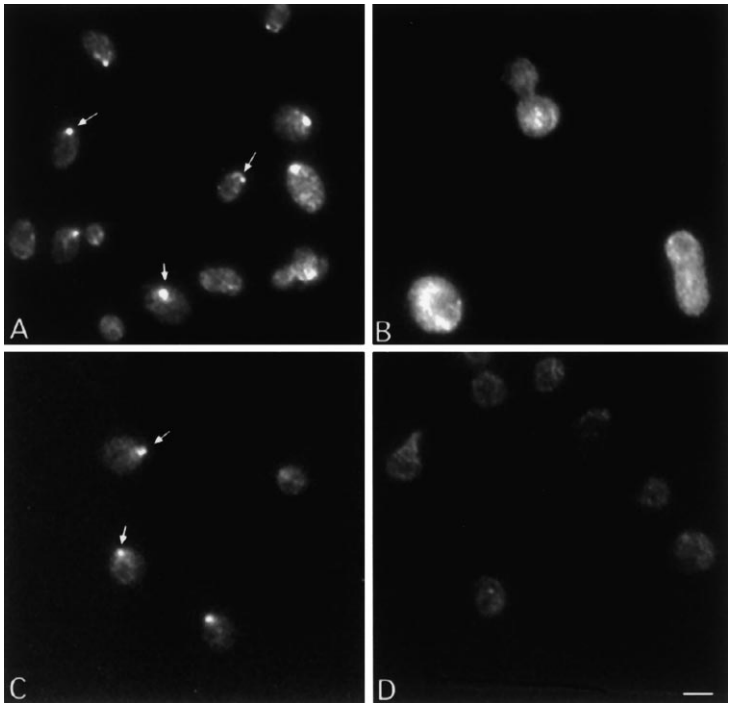

Fig. 3. The polarized localization of Sec4p immunofluorescence is lost in cells overexpressing the carboxy-terminus of Bmh2p. Wild-type cells (A), cells conditionally overexpressing the carboxy-terminus of Bmh2p (B) and cells with a temperature-sensitive mutation of Sec4p, *sec4-8*, grown at 25°C (C) or shifted to 37°C for 1 h (D) were all grown in galactose. Cells were then fixed in formaldehyde, the cell wall was removed and stained with a polyclonal antibody recognizing Sec4p to indicate active sites of growth (indicated by arrows). Note the lack of a localized fluorescent signal for Sec4p in B. Scale bar: 1 μm.

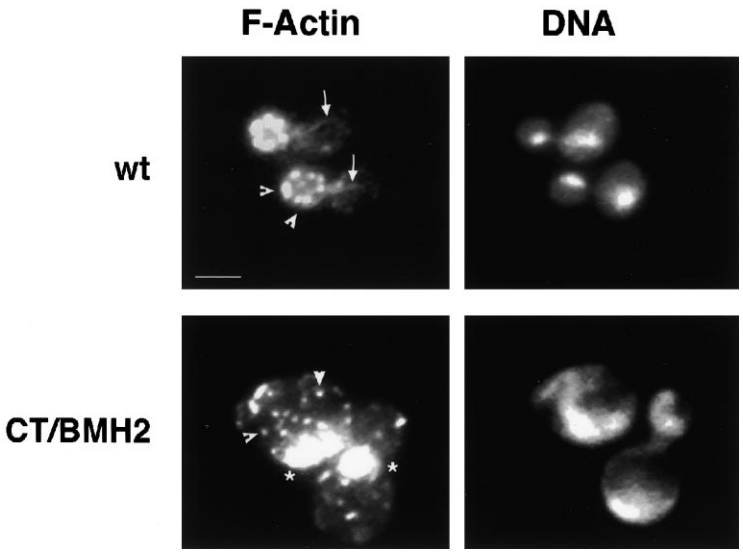

Fig. 4. The actin cytoskeleton is disrupted upon overexpression of CT/BMH2. Wild-type cells (wt) and cells overexpressing CT/BMH2 were pre-grown in rich medium containing glycerol and then induced in galactose-containing medium for 10 h. Cells were then fixed in formaldehyde and stained with TRITC-phalloidin to visualize filamentous actin (column labelled as F-Actin) and co-stained with DAPI (column labelled as DNA) to visualize nuclei. Arrow heads indicate actin patches that are localized in wild-type cells or became delocalized upon overexpression of CT/BMH2. Small arrows indicate actin filaments present in wild-type cells. The asterisks show the localization of actin clusters. Scale bar: 1 μm.

Potential vesicle targeting defects were therefore analyzed by indirect immunofluorescence with a vesicle marker, the rab-like GTPase Sec4p [36].

Sec4p staining in wild-type cells marks the sites of exocytosis according to the respective stage of the cell cycle at the bud tip or the neck region [36] and is thus a reliable indicator of polarized secretion. Cells harboring the CT/BMH2 allele (Fig. 3B) and the parental cells (Fig. 3A) were grown in galactose before indirect immunofluorescence for Sec4p was performed. The majority of cells overexpressing CT/BMH2 failed to show localized Sec4p staining at the bud tip or the neck region (Fig. 3B). Localized Sec4p staining was only visible in 18.8% of cells ($n = 841$) overexpressing CT/BMH2, whereas 79.7% of the parental cells ($n = 675$) showed a correctly localized Sec4p fluorescence signal at the bud tip or the neck. Furthermore, Sec4p fluorescence was prominent throughout the entire cell after CT/BMH2 overexpression, consistent with an even distribution of vesicles throughout the cells. Control experiments in which either the secondary antibody was omitted (data not shown) or a *sec4-8* temperature-sensitive mutant was used (Fig. 3C,D) revealed a marked reduction in the fluorescence signals (Fig. 3D), consistent with previously reported observations [36,37]. We therefore conclude that overexpression of CT/BMH2 causes a loss of localized Sec4p staining but not a complete loss of the Sec4p fluorescence signal. This loss of a localized fluorescence signal could not be attributed to a decrease in the levels of Sec4p expression since lysates of parental cells and cells overexpressing CT/BMH2 showed equal levels of Sec4p as judged by Western blot analysis (data not shown). This suggests that Sec4p-containing vesicles were randomly distributed upon CT/BMH2 overexpression. Our data are thus indicative of a vesicle targeting defect.

Given the previous indication of a potential role of 14-3-3 proteins on the actin cytoskeleton [18], we next analyzed the organization of the actin cytoskeleton in cells overexpressing CT/BMH2 (Fig. 4A). Staining of filamentous actin with TRITC-phalloidin in cells expressing the dominant-negative allele showed the occurrence of large actin clusters that frequently appeared in the neck region (Fig. 4B). Furthermore, actin patches, if existent, were distributed throughout the cell, while actin cables were no longer visible. These data demonstrate that overexpression of CT/BMH2 causes defects in the organization of the actin cytoskeleton. As filamentous actin in yeast may serve as tracks for vesicle transport from the Golgi to the plasma membrane, the observed changes in the actin cytoskeleton resulting from CT/BMH2 overexpression may explain why these cells display vesicle targeting defects.

4. Discussion

Here, we have analyzed the effect of dominant-negative alleles of 14-3-3 proteins on vesicle trafficking from the *trans*-Golgi network to the plasma membrane and on the actin organization in a highly polarized organism, the budding yeast *S. cerevisiae*. Our data show that domains in the amino- and carboxy-terminal regions of one 14-3-3 homologue, Bmh2p, but not of Bmh1p, become dominant-negative for growth upon overexpression. Analysis of the phenotype of cells overexpressing the carboxy-terminus of Bmh2p revealed that bulk invertase secretion remained unaltered, whereas the polarized localization of Sec4p, characteristic for vesicle transport to sites of active growth, was lost. In addition, the organization of the actin cytoskeleton was severely altered. These data indicate that at least one 14-3-3 yeast homologue is involved in pathways controlling actin organization and thereby in the targeting of post-Golgi vesicles to specific sites at the plasma membrane.

Our data demonstrate an in vivo function for the amino- and carboxy-termini of Bmh2p, which after overexpression cause an inhibition of cell growth. As the amino-terminus of

mammalian 14-3-3 proteins is known to be required for the formation of dimers [38–41] and the fragments used in this study are likely to encompass these dimerization domain as judged by amino acid homology (65% amino acid identity between Bmh2p and rat zeta in this region), the growth inhibition occurring upon NT/BMH2 overexpression provides a first indication that dimerization of 14-3-3 proteins is of functional importance in vivo.

The carboxy-termini of Bmh1p and Bmh2p may be involved in binding to downstream partner proteins as suggested for mammalian 14-3-3 isoforms [31–33]. Growth inhibition upon overexpression of CT/BMH2 is therefore indicative of a blockade of the downstream signaling pathway required for cell function. However, we cannot rule out that Bmh1p also functions in a similar pathway as we were not able to judge by Western blot analysis the relative expression levels of the overexpressed Bmh1p and Bmh2p fragments.

In CT/BMH2 overexpressing yeast cells, we did not see a defect in bulk invertase secretion but found a loss of targeted vesicle transport to the plasma membrane. This may be attributed to a breakdown of the actin cytoskeleton as indicated by the presence of actin clusters and the occurrence of bundles of microfilaments. Numerous reports support the idea that the actin cytoskeleton provides a track for polarized vesicle transport in yeast [26,43,44]. Temperature-sensitive mutations of actin are defective in polarized transport when grown at the restrictive temperature [34] and disruption of filamentous actin by the toxin latrunculin A results in a loss of vesicle polarization as revealed by a loss of Sec4p staining at the bud tip [26]. Depending on the cell type and the predominant role of the actin cytoskeleton, disruption of 14-3-3 function may produce different effects on the secretory process. In polarized cells such as yeast, the major role of the actin cytoskeleton in secretion may be to direct vesicles to sites of active growth [26,44]. Consequently, localized secretion directly depends on 14-3-3 function. In contrast, in chromaffin cells, where a dense cortical network underlies the plasma membrane and where secretory granules are largely stored in the cytoplasm, the transport of these granules to the plasma membrane depends on the removal of this cortical actin barrier [42,45,46]. Hence, proteins removing this barrier, like actin severing proteins and 14-3-3 proteins, will facilitate secretion in this type of cells [18,47,48].

It is tempting to speculate that modulation of the actin cytoskeleton by 14-3-3 proteins may play a general role in the establishment of cell polarity including cellular morphogenesis. One potential pathway through which 14-3-3 proteins may conceivably act to modulate the actin organization in yeast is the MAP kinase pathway. Bmh1p and Bmh2p have recently been shown to bind to Ste20p, one component of the yeast MAP kinase pathway involved in morphogenesis during pheromone response signaling [23]. It remains to be shown whether this or other pathways mediate 14-3-3 protein regulation of the actin cytoskeleton and of secretory processes.

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