**BED1**, a Gene Encoding a Galactosyltransferase Homologue, Is Required for Polarized Growth and Efficient Bud Emergence in *Saccharomyces cerevisiae*

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**Abstract.** The ellipsoidal shape of the yeast *Saccharomyces cerevisiae* is the result of successive isotropic/apical growth switches that are regulated in a cell cycle-dependent manner. It is thought that growth polarity is governed by the remodeling of the actin cytoskeleton that is itself under the control of the cell cycle machinery. The cell cycle and the morphogenesis cycle are tightly coupled and it has been recently suggested that a morphogenesis/polarity checkpoint control monitors bud emergence in order to maintain the coupling of these two events (Lew, D. J., and S. I. Reed. 1995. *J. Cell Biol.* 129:739–749). During a screen based on the inability of cells impaired in the budding process to survive when the morphogenesis checkpoint control is abolished, we identified and characterized *BED1*, a new gene that is required for efficient budding. Cells carrying a disrupted allele of *BED1* no longer have the wild-type ellipsoidal shape characteristic of *S. cerevisiae*, are larger than wild-type cells, are deficient in bud emergence, and depend upon an intact morphogenesis checkpoint control to survive. These cells show defects in polarized growth despite the fact that the actin cytoskeleton appears normal. Our results suggest that Bed1 is a type II membrane protein localized in the endoplasmic reticulum. *BED1* is significantly homologous to *gma12*+, a *S. pombe* gene coding for an α-1,2-galactosyltransferase, suggesting that glycosylation of specific proteins or lipids could be important for signaling in the switch to polarized growth and in bud emergence.

The ellipsoidal shape of the yeast *Saccharomyces cerevisiae* reflects cell cycle-regulated polarized growth. At specific times during the cell cycle, cell growth is either isotropic or polarized toward the bud (for review see Lew and Reed, 1995b). A correlation between local deposition of new cell wall components and actin localization has been established (Adams and Pringle, 1984; Kilmartin and Adams, 1984), leading to the proposal that actin directs secretory vesicles to specific regions of the plasma membrane to allow localized cell surface growth during bud initiation and bud growth. During most of the G1 phase, growth is isotropic and cortical actin patches are delocalized throughout the cell. The attainment of a critical cell size and concomitant execution of START lead to the formation of an actin ring at the pre-bud site and the orientation of actin filaments toward this site. Subsequent to START, growth is almost completely restricted to the emerging bud. During bud growth, cortical actin patches are localized to the bud. Initially, bud growth occurs primarily at the distal tip. At some point, though, there is a switch to isotropic growth first in the bud, then also transiently in the mother cell at mitosis. At cytokinesis, the actin cytoskeleton is reorganized and actin patches are relocated to the mother-daughter neck where the cell wall is modified for cell separation. The mechanisms by which actin mediates polarized secretion are not well understood, but it has been shown that cortical actin patches are associated with the cell surface through an invagination of the plasma membrane (Mulholland et al., 1994) and it has been suggested that components of the secretory pathway (endoplasmic reticulum [ER] and Golgi) could be transported into the bud to direct localized growth presumably via an actin-dependent mechanism (Preuss et al., 1992).

A variety of proteins have been shown to be required for either bud emergence or for bud site selection (for recent reviews see Bretscher et al., 1994; Chant, 1994; Welch and Drubin, 1994). *CDC42*, encoding a small GTP-binding protein, and several genes encoding its regulators are involved in bud emergence; cells mutated in these genes arrest as large unbudded cells with a disorganized actin cytoskeleton and delocalized chitin. The *BUD* genes, along with *CDC24* and *RSV167* are involved in the selection of the bud site.

In *S. cerevisiae*, the budding cycle is tightly coupled to the central events of the cell cycle. Upon completion of the primary G1 restriction event known as START, when the
mother cell has reached a critical size, bud emergence and S phase are initiated (Nasmyth, 1993; Reed, 1992). Moreover, the dramatic changes of actin organization and the isotropic/apical growth switches observed during the cell cycle have been shown to be triggered by the different forms of the Cdc28 kinase that constitute the cell cycle clock (Lew and Reed, 1993). Furthermore, it has been recently suggested that growth polarity or bud emergence are monitored to ensure that mitosis does not occur before a bud is produced to receive the daughter nucleus (Lew and Reed, 1995a). The impairment of growth polarity either by mutation or external stimuli such as osmotic shock is detected and results in a G2 delay. This morphogenesis/polarity checkpoint control is mediated via a partial inhibition of transcription of the mitotic cyclin genes CLB1 and CLB2 and also through a more direct inhibition of the mitotic form of the Cdc28 kinase via negative regulatory phosphorylation of Tyr19 of Cdc28 (Lew and Reed, 1995a). This conserved tyrosine has been shown to be the target of negative regulatory phosphorylation for a number of different cyclin-dependent kinases.

In this paper, we describe the identification and the characterization of a new gene called BED1 (which stands for Bud Emergence Delay) that is required for efficient polarized growth and is important for bud emergence. The bed1-1 mutation was isolated based on synthetic lethality with overexpression of mitotic cyclins, a phenotype that occurs because bed1 mutant cells depend on the morphogenesis/polarity checkpoint which overexpression of mitotic cyclins overrides. We have shown that the Bed1 protein is an integral membrane protein localized in the endoplasmic reticulum. This protein shares homology with a previously described S. pombe α-1,2-galactosyltransferase.

Our results suggest that glycosyl modification could play a role in regulating growth polarity and bud emergence.

Materials and Methods

Yeast Strains, Media, and Growth Conditions

All strains used in this study were derivatives of BP264-15DU: MATa ade1, his2, leu2-3,112, trp1-1, ura3Dns (Richardson et al., 1989). The relevant genotypes of strains used in this study are shown in Table I. Yeast cultures were grown at 30°C in YEP (1% yeast extract, 2% bactopeptone, 0.005% adenine, 0.005% uracil) supplemented with 2% glucose (YPEP), raffinose (YPEP), or galactose (YPEG). Genes under control of the GAL1 promoter were induced by the addition of 2% galactose to a mid-log phase culture (YPEP) for 4 h.

Identification and Molecular Characterization of the BED1 Gene

A strain carrying a GAL1:CLB2 allele (GY-1) was mutagenized by ultraviolet radiation (70% death) on YEPD plates (GAL1 promoter repressed) and incubated at 30°C for 2 d. The colonies were then replicated to YEPG (GAL1 promoter induced). Out of 25,000 colonies screened, 20 were unable to grow on galactose. Based on the level of Clb2 overexpression, we discarded 11 candidates that showed low levels of Clb2 protein after 4 h galactose induction of the GAL1:CLB2 allele, presumably because the mutations affected the galactose pathway. The nine remaining candidates were then crossed to the wild-type 15D strain and the resulting tetrads were analyzed to show that, for eight of them, the lethality was associated with overexpression of Clb2. The mutant strains were backcrossed to a MATa-GAL1:CLB2 strain (GY-101) and the diploid strains were then induced to sporulate and meiotic asci dissected to verify that the lethality on YEPG was due to a single mutation and that the mutations were not localized to the GAL1:CLB2 locus. A complementation analysis with the 8 remaining candidates showed that they belong to 6 different complementation groups, 2 of them with 2 alleles. We analyzed in greater detail one of them, that contains one allele, bed1-1.

The BED1 gene was cloned by complementation of the lethality of the strain GY-159 (GAL1:CLB2-bed1-1) on YEPG plates with a YCp50 based genomic yeast DNA library (Rose et al., 1987). The screening of 15,000 transformants (the equivalent of 10 genomes) yielded the plasmid pR159.1 times and the plasmid pR159.5 a single time, containing inserts.

1. Abbreviations used in this paper: BED, bud emergence delay; FOA, fluororotic acid; ORF, open reading frame.

Table I. Yeast Strains

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>15Daub</td>
<td>MATa-ade1-his2-leu2-3,112-trp1-1*-ura3Dns-bar1Δ</td>
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<tr>
<td>DLY-005</td>
<td>MATaα</td>
<td>D. J. L.</td>
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<td>MATa-GAL1:CLB2(LEU2).bed1-1</td>
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<tr>
<td>GY-755</td>
<td>MATaα-BED1::BED1(LEU2)</td>
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* S. I. R., Steven I. Reed; D. J. L., Daniel J. Lew.
of 12 kb and 9 kb, respectively (see Fig. 7A). These two DNA fragments had an overlap of a region of 2.7 kb that was able to rescue the lethality of the strain GY-159 on YEPD medium. This region was sequenced on both strands using an automated sequencing system (Applied Biosystems, Foster City, CA).

A diploid strain BED1/bedl-1::BED2(LEU2) (GY-650, see below) was constructed, induced to sporulate, and 48 tetrads were analyzed and 47 parental ditype (PD); 1 tetratype (TF) were observed demonstrating a tight linkage between bedl-1 and LEU2 loci (~1 cm). We also created a diploid strain BED1::BED1(LEU2)/bedl-1 (GY-651; see below), dissected 23 tetrads and 23 PD: 0 NPD: 0 TT were recovered, indicating again that the gene cloned by complementation was likely to correspond to the BED1 locus defined mutationaly.

Plasmids

The 2.7-kb DNA fragment defined by the region of overlap between plasmids pR159.1 and pR159.5 was subcloned into pBluescript (Stratagene, La Jolla, CA) and the BEDI gene was disrupted by replacing an 0.8-kb XbaI fragment within the coding region with a HindIII DNA fragment containing the URA3 gene to give the plasmid pBS159:URA3C (see Fig. 1 B).

The BEDI ORF was amplified by PCR using primers containing BamHI site before the STOP codon (primers: 5'-CCGTGATCACATTGTCTAGTGTACCT-3'; 5'-CCCCGGATCCATTA...-3') cloned into the BamHI site of YIpBED1::URA3 [HA]3X, respectively. The PCR products were sequenced on both strands using an automated sequencing system (Applied Biosystems, Foster City, CA).

Strain Construction

Strains were constructed according to standard genetic procedures (Sherman et al., 1982) except that transformations of yeast cells were performed as described by Eile (1992). The strain GY-381, disrupted for the BEDI gene, was obtained by transformation of a wild-type haploid strain with the plasmid pBS159:URA3C digested with StuI. The disruption was verified by Southern analysis (not shown).

The LEU2 marked BEDI gene was inserted at the bedl-1 locus by transformation of a Mata::bedl-1 strain (GY-488) with the plasmid YIpBED2::BEDI digested with NheI; this strain (GY-489) was then crossed to the Mata::bedl-1 strain by transformation of a wild-type LEU2 locus in 15Daub strain (GY-755) and thence to a Mata::bedl-1 strain (GY-488). The strain GY-650 and GY-651 were used to ascertain that the cloned BEDI gene was mutated in the bedl-1 strain.

The strains GY-711, GY-713, GY-721, and GY-723 were obtained by transformation of GY-381 with YIpBED2.BEDI, YIp2BED2::BEDI, and YIpBED2::BEDI as previously described (Grandin and Reed, 1993; Amon et al., 1994), thus avoiding deleterious effects to the cell. We took advantage of these observations to implement a screen for mutants able to tolerate high levels of Cib2 expression (see Materials and Methods). Cells carrying an inducible CLB2 allele under the control of the GALI promoter is not lethal in S. cerevisiae. The cells are only delayed in mitosis, presumably because the mitotic cyclin destruction pathway is sufficient to overcome the resultant elevated cyclin levels (Stueland et al., 1993; Amon et al., 1994), thus avoiding deleterious effects to the cell. We took advantage of these observations to implement a screen for mutants able to tolerate high levels of Cib2 expression (see Materials and Methods). Cells carrying an inducible CLB2 allele under the control of the GALI promoter (GY-1) were mutagenized by UV irradiation and we selected mutants that were unable to grow on YEPP which induces the GALI::CLB2 allele. Two categories of mutations are expected from such a screen. First, cells affected in their ability to degrade mitotic cyclins will arrest in mitosis because they cannot overcome the accumulation of CLB2 leading to chronic activation of the mitotic cyclin destruction pathway. This results in cells that are unable to enter mitosis and are therefore arrested in the G1 phase of the cell cycle. Second, cells affected in their ability to degrade mitotic cyclins will arrest in mitosis because they cannot overcome the accumulation of CLB2 leading to chronic activation of the mitotic cyclin destruction pathway. This results in cells that are unable to enter mitosis and are therefore arrested in the G1 phase of the cell cycle.
totic form of Cdc28 kinase. This situation would be analogous to the lethality observed when a nondegradable form of Clb1 (Clb1Δ152) is overexpressed in wild-type cells (Ghiara et al., 1991). A second expected class of mutation conferring a defect in bud emergence or growth polarity can also be obtained with this screening method. Delays or blocks in generation of growth polarity or in bud emergence are detected by a morphogenesis checkpoint leading to a temporary G2 arrest (Lew and Reed, 1995a). This control can be overridden either by mutation of tyrosine 19 of the Cdc28 kinase or the overexpression of mitotic cyclins Clb1 or Clb2 (Lew and Reed, 1995a). Therefore mutations that delay budding or the generation of growth polarity could confer a lethal phenotype when CLB2 is overexpressed due to abrogation of the checkpoint. In this situation, cells dependent on the checkpoint would die with more than one nucleus as a result of mitosis occurring.

Figure 1. Overexpression of the mitotic cyclin Clb2 is lethal in a bed1-1 background. The FACS profiles of bed1::GAL1::CLB2 (GY-1) and bed1-1::GAL1::CLB2 (GY-159) strains were determined after 4 h induction with galactose.

Figure 2. Phenotype bed1 disruptant cells. (A) FACS analysis of bed1::URA3 cells. Wild-type (15 Daub), bed1::URA3 (GY-381) grown at mid-log phase in YEPD or YEPR, bed1::GAL1::CLB2 (GY-1) and bed1::URA3::GAL1::CLB2 (GY-382) induced at mid-log phase for 4 h with 2% galactose were analyzed by FACS as described in Materials and Methods. Note that the budding index was probably overestimated in bed1::URA3 sample because of the cell aggregation observed in these cells. (B) Morphology of bed1::URA3 cells. Phase-contrast micrographs of wild-type (15Daub) and bed1::URA3 (GY-381) cells grown in YEPD or YEPR. Magnification is the same for both strains. Bar, 10 μm.
A bed1::URA3 Strain Is Defective in Bud Emergence and the Morphogenesis Checkpoint Is Necessary for Its Viability

Although a GAL1:CLB2-bed1-1 strain was able to grow on dextrose medium (GAL promoter repressed) and not on galactose medium (GAL promoter induced), a bed1-1 strain was viable on both media. FACS analysis of nuclear DNA content of a strain overexpressing Clb2 in a bed1-1 background showed that a large fraction of the cells were arrested with a 4N DNA content (Fig. 1) while Clb2 overexpression in a wild-type background induced only a delay in mitosis (Stueland et al., 1993). Microscopic observation revealed that a large proportion of the cells had more than two nuclei (see below for detailed analysis of the bed1 phenotype). The BED1 gene was cloned, sequenced, and a null mutation in the BED1 gene was created by the one-step disruption method. The cloned gene was shown to be genetically linked to the BED1 locus (see Materials and Methods and below). We observed that the mutation and the targeted disruption conferred similar phenotypes and therefore used the bed1::URA3 strain to investigate the bed1 phenotype in detail. This strain was viable but showed a 30% reduction in growth rate in rich (YEPD) liquid medium compared to the isogenic wild-type strain. The cells were larger than wild-type and almost completely round, having lost the ellipsoidal morphology characteristic of S. cerevisiae (Fig. 2 B). Using a Coulter Channelizer, bed1::URA3 cells were shown to be 50% larger than wild-type cells in rich medium (the mean cell volume for bed1::URA3 cells was 64 fl vs 42 fl for wild-type cells). bed1::URA3 cells also had defects in cell separation, in that cultures contained clumps of aggregated cells which could not be completely disrupted by sonication (see Fig. 2 B). However, treatment with the cell wall-digesting enzyme Zymolyase gave single cells (not shown), indicating that the defect was in cell separation rather than cytokinesis. FACS analysis of bed1::URA3 cells showed an increase in the proportion of S/G2/M cells in an asynchronous culture in rich liquid medium. A small fraction of the cells scored as greater than 2N in DNA content, presumably because of the cell separation defect (Fig. 2 A). In wild-type cells, bud emergence is concomitant with the beginning of S phase as is illustrated by the correlation between the budding index and the percentage of cells that have entered or completed S phase (Fig. 2 A). These observations were suggestive of a defect in bud emergence conferred by the bed1::URA3 mutation.

To further characterize this phenotype, small G1 wild-type and bed1::URA3 cells were isolated by centrifugal elutriation, inoculated into fresh YEPD medium and execution of START, initiation of S phase, bud emergence, and nuclear division were followed (Fig. 3). As previously described (Lew et al., 1992), in the 15D wild-type background, completion of START was followed by S phase and bud emergence within 15–20 min. Nuclear division then occurred 45–50 min later. In bed1 disruptant cells,
completion of START occurred sooner than in wild-type cells, presumably because the elutriated population exhibited a larger size than the wild-type controls (not shown). S phase began normally 15–20 min after START but bud emergence was delayed by more than an hour. Nuclear division took place very rapidly after bud emergence, within 10–15 min, suggesting that the morphogenesis checkpoint delays mitosis only until a bud forms to receive the daughter nucleus. Only a small fraction of bed1::URA3 cells contained more than one nucleus whereas most of the cells overexpressing Clb2 in this background became multinucleated (Fig. 4). This is consistent with the fact that the overexpression of Clb2 or Clb1 is lethal in bed1-1 or bed1::URA3 cells (Fig. 1 A and Fig. 2 B; not shown). In a synchronized culture, these cells went through S phase and mitosis before bud emergence and died with more than two nuclei (not shown). The disruption of the BED1 gene in the context of the cdc28F19A18 mutation (where CDC28 is mutated so as to be no longer subject to negative regulatory phosphorylation) was not lethal but a large fraction of the cells were multinucleated (Fig. 4). It was difficult to quantify the percentage of multinucleated cells in these different strains because of the cell aggregation phenotype, but it was clear that more multinucleated cells were detected when Clb2 was overexpressed or Cdc28 was not phosphorylatable. It was also apparent that, in bed1::URA3 cells, nuclear division occurred very rapidly after bud emergence, in that daughter nuclei were observed even in very small buds while nuclear division took place in wild-type cells when buds were much larger (Fig. 4). We

![Figure 4](image-url)

**Figure 4.** The viability of bed1::URA3 cells depends on the integrity of the morphogenesis checkpoint control machinery. Nuclei were visualized by staining cells with DAPI: (A) wild-type (15Daub in YEPD); (B) bed1::URA3 (GY-381 in YEPD); (C) bed1::URA3-GAL1:CLB2 (GY-382 after a 4 h induction with 2% galactose in YEPR); (D) bed1::URA3-cdc28::LEU2-CDC28F19A18(TRP1) (GY-449 in YEPD). Note that the fields are not representative of the percentage of multinucleated cells in the different strains. Magnification is the same for all the strains. Bar, 10 μm.
concluded from these experiments that the \textit{BED1} gene was required for timely bud emergence, that the morphogenesis checkpoint is functional and that the viability of \textit{bed1::URA3} cells depends on the integrity of this checkpoint control machinery.

\textbf{\textit{bed1::URA3} Cells Have Defects in Polarized Growth Although Actin Is Properly Polarized}

The fact that \textit{bed1} mutant cells are delayed in bud emergence, are large, and have an unusually round morphology, suggested that they might be defective in polarized growth during the cell cycle. This type of phenotype is often associated with an inability to properly organize the actin cytoskeleton (see Discussion). We investigated this possibility by looking at actin localization by rhodamine-phalloidin staining of \textit{bed1} mutant cells. As shown in Fig. 5A, actin staining revealed a pattern similar to that observed in wild-type cells: actin rings were observed at the pre-bud site and after bud emergence, actin patches were found exclusively in the buds and actin cables were oriented toward the tips of the buds. Finally actin patches
were relocalized to the necks of the buds during cytokinesis. We also observed, as with wild-type cells, that actin patches were concentrated to the tip of the growth projection (shmoo) when bed1 mutants were treated with the mating pheromone α factor (not shown).

We then determined the timing of polarization of the actin cytoskeleton during the cell cycle in a synchronized culture (Fig. 3). bed1 mutant cells began to undergo actin polarization within 5 min after completing START, as in wild-type cells. We concluded, therefore, that actin was properly polarized at the appropriate time during the cell cycle, indicating that the bed1 phenotype does not result from an inability to reorganize the actin cytoskeleton at the G1/S phase boundary.

On the other hand, staining of bed1 mutants with calcofluor, a stain for chitin, which is normally found concentrated in the neck region of a budding cell and in “scars” on cells where previous buds were located, revealed that chitin was now completely delocalized and deposited at elevated levels (Fig. 5 B). One interpretation of this phenotype is that bed1 mutants are defective in polarized secretion required for proper localization of chitin synthase despite the fact that there is no obvious defect in polarization of the actin cytoskeleton.

Growth polarity can be directly visualized by pulse-labeling cells with FITC-ConA, which binds mannose residues from the cell wall and chasing in the absence of FITC-ConA (for a more detailed description of this method, see Lew and Reed, 1993). Exponentially growing wild-type and bed1::URA3 cells were pulse labeled and fixed after a chase of one generation equivalent in fresh medium. Daughter cells originating from labeled buds (cells with an unlabeled birth scar) were scored for staining patterns characteristic of isotropic growth (uniform staining; see, for example, cell 1 in Fig. 6 A) or apical growth (staining that fades out toward one end of the cell; see, for example, cell 2 in Fig. 6 A). As previously described (Lew and Reed, 1993), ~40% of the daughter cells from a wild-type population in YEPD medium exhibited polarized growth (169 out of 449 daughter cells). Among the cells exhibiting a pattern indicative of polarized growth, 15% showed a partial gradient of the staining where the pole of the cell was still detected (see cell 3 in Fig. 6 A; 27 out of 169). On the other hand, most of the bed1::URA3 daughter cells (~60%; 275 out of 452 daughter cells) exhibit a uniform staining indicative of completely isotropic growth (cell 4) and no daughter cells with completely unlabeled poles indicative of apical growth were observed. However, ~15% of these cells (66 out of

Figure 6. FITC-ConA pulse labeling of wild-type and bed1 cells. (A) Wild-type (DLY-005) and bed1 mutant (GY-647) diploid cells were pulse labeled with FITC-ConA for 15 min and after a chase with fresh medium during one generation time, cells were fixed and observed by fluorescence microscopy. Daughter cells generated from labeled buds with an unlabeled birth scar (indicated by an arrow) were scored for uniform staining indicative of an isotropic growth (cell 1), for staining that fades completely out toward the pole opposite from the unlabeled birth scar, indicative of polarized growth (cell 2) or for an incomplete or partial gradient toward the end of the daughter cell (cell 3). In the mutant population, daughter cells exhibiting uniform staining (cell 4) or an incomplete gradient toward one pole of the cell (cell 5) could be observed along with cells showing a weak uniform staining with brighter signal around the birth scar (cell 6). Bar, 10 μm. (B) Confocal microscopy was used to characterize the staining of the same cells in greater detail. Examples of staining indicative of polarized growth in wild-type cells are shown on the left. Mutant daughter cells exhibiting weak uniform staining with brighter staining around the birth scar or partial fade-out staining are shown on the right. Bar, 5 μm.
452) exhibited a partial fade-out staining with a decreasing gradient of the staining to one end of the cell but with the pole still labeled (see for example, cell 5 in Fig. 6 A) and ~25% (111 out of 452) showed a faint uniform staining with a zone of strong staining around the birth scar (see, for example, cell 6 in Fig. 6 A). We used confocal microscopy to analyze the fade-out staining in greater detail (Fig. 6 B). While no staining could be detected at the opposite pole of the unlabeled birth scar in wild-type cells, the end of bed1::URA3 daughter cells was always stained, suggesting that...
the apical growth, when it occurs, is defective or incomplete. Examination of cells with a stronger signal around the birth scar (cell 6 in Fig. 6 A) showed that the pattern is consistent with staining of a region very close to the scar itself but without any gradient toward the pole of the cell. Our interpretation is that growth cannot occur in this region for mechanical reasons and therefore the signal cannot be diluted during bud growth and appears as a narrow ring of heavily stained cell wall. The fact that we could still detect in some cells a partial gradient toward one pole of the cell (~15% of daughter cells) suggests that growth may be partially polarized at some point of the budding phase. Taken together, these results suggest that bed1::URA3 cells exhibit some growth polarity but that apical growth is not as efficient as in wild-type cells.

Molecular Characterization of the BED1 Gene: BED1 Shows Similarity to an S. pombe Gene Encoding an α-1,2-Galactosyltransferase

The gene encoding Bed1 was cloned by complementation of the GAL1:CLB2-bed1-1 mutant strain (GY-159) using a centromeric yeast genomic DNA library and two plasmids containing a 2.7-kb overlapping region were recovered (Fig. 7 A). We detected one large open reading frame (ORF) in this region with a capacity for encoding a protein of 393 amino acids (Fig. 7 B). Examination of the sequence of the BED1 gene showed a stretch of hydrophobic amino acids in the NH2-terminal portion of the ORF (residues 47 to 67; Fig. 7 B). The inferred structure of Bed1 was reminiscent of the organization of type II membrane proteins: a short NH2-terminal cytosolic domain, a unique hydrophobic transmembrane domain (15-20 amino acids) and a large luminal COOH-terminal domain (for review see High and Dobberstein, 1992).

BED1 had similarity to the recently cloned gma12+ gene of S. pombe (Chappell et al., 1994). The two proteins are similar in their predicted luminal domains: 4 regions that show ~30% identity and up to 70% similarity (Fig. 8). The structures of these two proteins are different in that, although they both have type II membrane protein structure, the cytoplasmic domain of Bed1 is larger (45 amino acids) than the corresponding domain of gma12p (only two amino acids amino terminal to the transmembrane domain). The gma12+ gene encodes an α-1,2-galactosyltransferase involved in the synthesis of the S. pombe wall which, in contrast to that of S. cerevisiae, contains glycoproteins with galactose residues. More recently, two putative ORF homologous to gma12p were detected on chromosome I of S. pombe. Therefore, gma12+ belongs to a highly conserved family of proteins that are conserved along their entire lengths in the fission yeasts S. pombe and S. octosporus (Fig. 8; Chappell, T., personal communication).

We also noticed that Bed1 is even more homologous to a previously unidentifed S. pombe partial ORF present adjacent to the vacuolar H+-ATPase, subunit B gene (Fig. 8). Analysis of the sequences available shows that the homology between Bed1 and this partial ORF is significantly greater than that between Bed1 and gma12p (see Fig. 8). The structure of this ORF is also more similar to the structure of Bed1 in that the putative cytoplasmic domain contains ~40 amino acids. However, the role of the protein represented in part by this ORF is not known. The possible implications of these homologies will be discussed below.

Bed1 Is a Type II Membrane Protein Localized in the Endoplasmic Reticulum

Since the analysis of bed1::URA3 cells suggested a role in polarized secretion and Bed1 had a predicted structure organization consistent with a membrane protein, we determined the intracellular localization of Bed1. We introduced a COOH-terminal triple influenza hemagglutinin (HA3X)-tagged version of Bed1 into bed1::URA3 cells. The HA-specific 12CA5 monoclonal antibody detected a

Figure 8. Homologies between BED1 and gma12+, an S. pombe gene encoding an α-1,2-galactosyltransferase. The search for sequences homologous to Bed1 was performed at the National Center for Biotechnology Information (NCBI) through the GENINFO (R) BLAST Network Service (Blaster) (Altschul et al., 1990). Four S. pombe ORFs were identified: gma12p (SPA12GATR; accession number Z09174), two ORFs on chromosome I (cds11 and cds13; accession number Z49811) and a previously unidentified partial ORF (spORF) located 5′ from the vacuolar H+-ATPase, subunit B gene (SPVATPB). The alignment of the 5 ORFs was established with the following rules: G=A=P=S; S=A=T; R=H=K; D=E; Q=N; M=I=L=V=F; F=W=Y.
doublet on Western blots of approximate molecular mass of 50 kD, in good agreement with the predicted molecular weight of the fusion protein (46 kD for Bed1 and 4 kD for the [HA]3X tag) (Fig. 9 A). The HA-tagged version of Bed1::URA3 was present in the low speed fraction (P13) enriched for endoplasmic reticulum, nuclear envelope, vacuoles, and plasma membrane. Kar2, a luminal protein of the ER (Rose et al., 1989), was also mainly present in this fraction, as expected. We also showed that, unlike Kar2, Bed1 was tightly associated with membranes: Bed1 remained membrane associated after treatment of the membrane fraction with carbonate pH 11.0 but not after treatment with detergents (not shown). Moreover, only the NH$_2$-terminal portion of Bed1 was sensitive to proteolysis by proteinase K when the protein was associated with intact membranes, suggesting that the first 45 amino acids are likely to be cytosolic in intact cells (not shown). Based on these criteria, we concluded that Bed1 is a type II integral membrane protein.

We were unable to detect Bed1::[HA]3X by immunofluorescence when the fusion protein was expressed under the control of its native promoter (not shown). Therefore, we constructed a strain containing the tagged protein under control of the constitutive GAP promoter (GY-718), yielding a 20-fold increase in the amount of Bed1 protein, as observed by Western blot (Fig. 9 A). Since it has been shown that, in some cases, overexpression of proteins of the secretory pathway can lead to a mislocalization due to saturation effects, we verified that Bed1 had the same fractionation profile when overexpressed as when it was expressed under control of its own promoter (Fig. 9 A). We observed an immunofluorescence staining pattern consistent with an ER localization of the protein: the signal was perinuclear with some extensions into the cytoplasm. This pattern was similar to the immunolocalization of Kar2, a luminal protein of the endoplasmic reticulum (Rose et al., 1989). We also observed that Bed1 colocalized with Kar2 in individual cells (not shown). These data taken together suggest that Bed1 is a type II integral membrane protein of the endoplasmic reticulum and support the hypothesis, based on homologies with gma12$, that Bed1 is a galactosyltransferase and that the switch to polarized secretion might require galactosyl modification of particular proteins or lipids during transit through the endoplasmic reticulum.

Discussion

The Bed1 gene was identified based on its involvement in bud emergence and polarized growth in S. cerevisiae. Although impaired, cells disrupted for Bed1 are viable, suggesting either that this gene encodes an important but nonessential function, or that Bed1 is redundant with another related gene. Bud emergence was strongly delayed, occurring only 1 h after initiation of S phase in bed1::URA3 cells while these two events were tightly coupled in wild-type cells. On the other hand, nuclear division took place immediately after bud emergence, indicating a tight coupling between these two events in bed1 mutant cells. However, bed1 cells became multinucleate when the mitotic form of Cdc28 was hyperactivated by either the overexpression of the mitotic cyclins Cln1 or Cln2 or by mutation of Tyr19, the regulatory phosphorylation site of Cdc28. Lew and Reed (1995a) have shown that, while the G2 delay induced by defects in growth polarity or budding can be completely abolished by Cln1 or Cln2 overexpression, the cdc28FTR mutation only reduces the delay but cannot eliminate it completely. This accounts for the observation that a bed1::URA3-cdc28FTR/Al8 strain is viable while overexpression of Cln2 in a bed1::URA3 background is lethal. Taken together, these observations suggest that the viability of bed1 cells depends on the morphogenesis checkpoint machinery which is able to delay mitosis in the absence of budding in order to maintain the coordination between the nuclear division cycle and the budding cycle. In fact, the dependence of the bed1 mutant on the morphogenesis checkpoint for survival is the most convincing demonstration of the importance of this regulatory system in the yeast life cycle. Finally, cells disrupted for Bed1 exhibited morphological aberrations, losing the ellipsoidal shape characteristic of S. cerevisiae and being larger than wild-type cells. In addition, bed1 cells had defects in cell separation as indicated by a tendency to form aggregates in liquid medium.

The delayed bud emergence, the morphological and morphogenetic phenotypes observed in bed1::URA3 cells (increased size, round cell shape, and delay in bud emergence) could be a result of defects in secretion or the generation of growth polarity by analogy with other morphogenesis mutants (for reviews see Bretscher et al., 1994; Welch and Drubin, 1994). Disorganization of the actin cytoskeleton and delocalized deposition of chitin are usually phenotypically coupled, presumably because proper actin function is required for polarized secretion and therefore for budding. In bed1::URA3 cells, actin polarization after START and actin reorganization at cytokinesis occurred on schedule. Surprisingly, however, chitin deposition was greatly increased and completely delocalized, indicating that bed1 cells have defects in polarized growth. Moreover, a more direct evaluation of growth polarity by in vivo pulse labeling with FITC-ConA showed that bed1::URA3 cells are defective in the most polarized form of growth, growth directed to the bud tip or apical growth. Some residual growth polarity was observed, explaining why bed1::URA3 cells are viable and suggesting that another gene redundant with Bed1 might be responsible for this activity. From these observations, we suggest that bed1 cells are impaired in directing secretory vesicles to the bud site but not in secretion per se, and that Bed1 is part of a pathway that is downstream or parallel to the actin pathway; both pathways being necessary for proper delivery of secretory vesicles to the bud site during bud emergence and to the bud neck during cytokinesis and cell separation.

A role in polarized secretion is also consistent with the intracellular localization of Bed1. Crude fractionation of whole cell lysates showed that Bed1 was present in a low speed fraction containing endoplasmic reticulum, vacuoles, nuclear envelope, and plasma membranes. Biochemical experiments indicated that Bed1 was an integral type II membrane protein with a small NH$_2$-terminal cytoplasmic
Figure 9. Bed1 is a membrane protein localized in the endoplasmic reticulum. (A) Bed1 is present in an ER-enriched fraction. Total protein extracts (TOTAL) containing intact membrane structures were subjected to a crude fractionation procedure: the P13 fraction is enriched for membranes from the endoplasmic reticulum, the vacuoles, the plasma membrane and the nuclear envelope; the P100 fraction is enriched for Golgi membranes; the S100 fraction contains soluble proteins. The Bed1[HA]3X protein was detected by Western blot with the 12CA5 mouse monoclonal antibody and Kar2 with a rabbit polyclonal antibody. Note that twice as much volume of GY-723 (BED1[HA]3X) was loaded compared to GY-718 (GAP:BED1[HA]3X). It was estimated by densitometric scanning of different exposures of the Western blots that 40% of Kar2 was in the P13 fraction, 20% in the P100, and 20% in the S100 fraction. The same kind of measurement gave for Bed1 90%, 10%, and less than 1% in fractions P13, P100, and S100, respectively. (B) Immunolocalization of Bed1. GY-718 (containing GAP:BED1[HA]3X) and GY-716 (containing GAP:BED1 as a negative control) cells grown in YEPD were stained with DAPI to visualize the nuclei and at the same time with the 12CA5 antibodies to detect the Bed1 fusion protein. Magnification and exposure time are the same for both strains. Bar, 5 μm.
domain and COOH-terminal luminal domain. The predicted structure of the protein was in agreement with this conclusion since Bedl contained a putative hydrophobic transmembrane domain in the NH2-terminal portion of the protein (amino acids 47–67). We were not able to detect an epitope-tagged version of the protein expressed from its own promoter by immunofluorescence because of low levels of expression but a staining pattern suggesting an ER localization for Bedl was obtained when the tagged protein was overexpressed. We showed however that the behavior of Bedl in the fractionation procedure we used was not affected by overexpression from the constitutive GAP promoter. Taken together, these two different approaches suggested that Bedl is an integral type II membrane protein of the ER.

We discovered recently that the BED1 gene was independently cloned as SLC2 (Karpova et al., 1993). slc2 mutants were identified during a screen designed to identify mutations synthetically lethal with a disruption of CAP2, a gene involved in actin cytoskeleton organization (Karpova et al., 1993). The phenotype associated with the slc2-107 allele is different from that described here: the actin cytoskeleton is disorganized in a strain carrying the slc2-107 allele and this strain is thermosensitive while bedl::URA3 cells have a normal pattern of actin polarization and are not temperature sensitive (at least up to 37°C, not shown). This could be explained either by strain background differences or by the fact that the slc2-107 mutation is semidominant, suggesting that this mutation might be associated with a gain-of-function. More recently, the sequence of the BED1 gene appeared twice in the Genbank database: it was detected during the sequencing of chromosome IV and BED1 is identical to MNN10 (accession number L42540). mnn mutants were isolated as mutants that have aberrant carbohydrate structures in the cell wall; most of the gene products are thought to be involved in mannosylation of proteins but some could be involved in more general functions of the secretory pathway that might affect mannosylation indirectly (for review see Hercovics and Orlean, 1993). This latter hypothesis is consistent with our results.

BED1 was found to be similar to 4 ORFs in S. pombe, gma12+ and 2 of its homologues and a previously unidentified ORF we called spORF. The homology between Bedl and gma12p/cds11/cds13 was particularly significant over 4 regions in the luminal portion of these proteins (~30% identity and 65% similarity). The gma12p protein has been shown to be an α-1,2-galactosyltransferase (Chappell et al., 1994). Several galactosyltransferase activities have been detected in S. pombe (Chappell et al., 1994; Ballou and Bal lou, 1995) and the gma12+ gene belongs to a large family of related genes in S. pombe (Chappell, T., personal communication). Although the structures of the two proteins are clearly similar, Bedl has a larger cytoplasmic domain. On the other hand, this domain is comparable in size with the corresponding domain of spORF and, furthermore, comparison of the available sequences of spORF and Bedl (see Fig. 7) showed that the degree of similarity between Bedl and spORF was higher than between Bedl and gma12p (~40–50% identity and 70% similarity). We conclude from these observations that spORF is more likely than gma12+ to be the homologue of BED1 in S. pombe. Indeed, gma12+ could not complement the morphological defects observed in bedl cells, although it was shown to be enzymatically active in vitro in extracts from S. cerevisiae cells overexpressing gma12p under the control of the GALI promoter (not shown). Moreover, localization of gma12p in the Golgi apparatus (Chappell et al., 1994) is distinct from that of Bedl. Finally, gma12p is involved in bulk modification of proteins of the cell wall, a phenomenon particular to S. pombe, that has not been detected in S. cerevisiae; we propose that the role of Bedl is more specific (see below).

It was surprising to find homology between Bedl and a galactosyltransferase since, to our knowledge, no galactosyl modifications have been described for glycoproteins or glycolipids in S. cerevisiae. We were unable to detect any galactosyltransferase activity in extracts from wild-type cells or from cells overexpressing Bedl under conditions where ectopically overexpressed gma12p showed significant activity (not shown). This assay, based on conditions described for gma12p (Chappell and Warren, 1992), is somewhat restrictive and does not rule out the possibility that Bedl could be a galactosyltransferase that cannot use α-methylmannoside or α-methylgalactoside as an acceptor. Furthermore, the idea that Bedl is a galactosyltransferase is in conflict with the fact that no phenotype has been described in association with disruption of the GAL10 gene encoding UDP-glucose 4-epimerase, the enzyme responsible for interconversion of UDP-glucose and UDP-galactose. We verified that the disruption of this gene in our genetic background did not confer a morphological phenotype similar to that of bedl::URA3 cells when grown on glucose medium (not shown). An alternative possibility is that another epimerase is present in S. cerevisiae and is responsible for the production of UDP-galactose from UDP-glucose for the purpose of galactosyl modification of specific proteins or lipids. Such modification targeted to specific protein or lipid species could have escaped detection in analysis of bulk glycoproteins or glycolipids. This raises the intriguing possibility that specific galactosyl modification may be involved in signaling the isotropic to polarized switch in secretion. We are currently investigating using a PCR approach the hypothesis that S. cerevisiae contains other glucose-4-epimerase(s). Alternatively, Bedl may catalyze a different glycosyl modification although, based on precedent, this is unlikely: high levels of structural homology have been detected only between enzymes that catalyze analogous glycosylation reactions (Kleene and Berger, 1993).

A simple model to explain the role of Bedl in polarized growth and therefore in bud emergence is that Bedl is involved in modification of an effector protein that controls the targeting of the secretory vesicles via the actin cytoskeleton in the context of the switch from isotropic to polarized growth at the G1/S phase boundary. In an alternative model, Bedl might catalyze a modification that leads to local reorganization of the membrane and/or the cell wall at the bud site, allowing vesicles to fuse more efficiently with the plasma membrane. Elucidation of the function of Bedl will require the identification and characterization of its target(s).

It is interesting that bedl mutant cells show defects in polarized secretion and that cell division occurs when buds are unusually small (Fig. 4). One interpretation of this ob-
In such circumstances, significant growth cannot occur until a bed is initiated without highly polarized secretion but that, under such circumstances, significant growth cannot occur until a bud becomes an autonomous cell. Thus, whereas bud growth is normally integrated as a phase of each cell cycle, in bed1 cells, it may be pushed forward to the subsequent cell cycle.

In summary, we have characterized a new gene that is required for efficient bud emergence and apical growth. Cells carrying a disrupted allele of BED1 are viable but depend upon a morphogenesis checkpoint to survive. The defects observed in bed1 cells indicate that this protein is in a pathway downstream or parallel to the actin polarization pathway in mediating the switch from isotropic to polarized growth. The fact that Bed1 shares significant homology with an S. pombe galactosyltransferase suggests that glycosylation might have an important signaling role in this process.

We thank Fred Cross, Mark Johnston, Dave Stuart, Martin Snider, and Henar Valdivieso for generous gift of plasmids and Mark Rose for the anti-Kar2 serum; George Klier for help with the confocal microscopy; Tom Chappell, John Cooper, and Martin Snider for sharing results before publication. Danny Lew, Dave Stuart, and Curt Wittenberg are acknowledged for the critical reading of this manuscript. G. Mondésert would like to thank the members of the Reed, Wittenberg, and Russell Laboratories for fruitful interactions, especially Marie-Noelle Simon and Eric Bailly for daily discussions and encouragement and Danny Lew for discussions on morphogenesis.

G. Mondésert acknowledges fellowships from the European Molecular Biology Organization and the Human Frontiers in Science Program. This research was supported by a U.S. Public Health Service Grant (GM38328) to S. I. Reed.

Received for publication 23 June 1995 and in revised form 17 October 1995.

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