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High-Level Expression of Endogenous Acid Phosphatase Inhibits Growth and Vectorial Secretion in *Saccharomyces cerevisiae*

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Abstract The secretion pathway of *Saccharomyces cerevisiae* was challenged by constitutively overexpressing plasmid-encoded acid phosphatase, a secreted endogenous glycoprotein. A 2-μm-based multicopy plasmid carrying the coding sequence of acid phosphatase under the control of a truncated variant of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter was used for expression. Selection for the promoterless *dLEU2* marker leads to a growth arrest. This is not per se due to leucine starvation, but due to intracellular accumulation of highly glycosylated enzymatically active acid phosphatase. Immunofluorescence and cytological analysis indicate that intracellular accumulation of acid phosphatase occurs in a subpopulation of cells. By Ludox-AM density centrifugation, these cells can be enriched on the basis of their higher density. The dense accumulating cells have a higher average plasmid copy number and produce more acid phosphatase than non-accumulating cells of low density. These cells are defective in directed secretion and bud formation, therefore can no longer grow and show dramatic changes in cell morphology. We suggest that the secretion pathway in these cells is overloaded with the high level of acid phosphatase leading to a shutdown in vectorial secretion, subsequently to a standstill in growth and to the intracellular accumulation of further expressed acid phosphatase. We have indications that accumulation of acid phosphatase occurs in the late Golgi, suggesting a limitation of the overall secretion at this stage.

Key words: growth arrest, secretion, yeast, plasmid copy number, Berkeley bodies, Golgi apparatus, overexpression, vectorial transport

The protein secretion pathway appears to be well conserved in higher as well as in lower eukaryotes such as *Saccharomyces cerevisiae.* Many of the molecular components involved in the different steps have been identified [Pryer et al., 1992]. The knowledge about individual components, and their function does not necessarily lead to an understanding of the overall performance of the secretion pathway and does not explain obvious differences between higher and lower eucaryotes [Burgess and Kelly, 1987; Preuss et al., 1992].

The yeast *S. cerevisiae* secretes numerous polypeptides via a nonselective bulk flow to the cell surface [Pfeffer and Rothman, 1987]. Efficient transport of molecules to the bud region is required for growth of yeast [Field and Schekman, 1980; Novick and Schekman, 1979; see for review, Drubin, 1991; Chant and Pringle, 1991]. Components of the cytoskeleton are involved in this directed transport. Actin, which determines the emergence of the bud, is shown to play an important role in organization and assembly of cell surface growth at the bud [Kilmartin and Adams, 1984; Adams and Pringle, 1984; Novick and Botstein, 1985; Chant and Pringle, 1991].

The yeast system has also been widely used for the expression and secretion of heterologous proteins [Moir, 1989; Romanos, 1992; Hinnen et al., 1989]. Secretion allows the convenient placement of proteins outside the cell. Particularly the yeast secretion pathway offers the op-

Abbreviations used: endoplasmic reticulum (ER); kilobase pair (kbp); acid phosphatase (APase).

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portunity to obtain properly folded and biologically active molecules. The efficiency of the secretion process is an important factor for high level expression of secreted proteins. In comparison to filamentous fungi and higher eukaryotic cells the total amount of secreted protein in yeast is rather low, raising the question whether a step in secretion is rate-limiting. Indeed, the issue of secretion capacity is controversial [Moir, 1989]. It seems necessary to consider secretion of genuine yeast (homologous) proteins separate from secretion of foreign (heterologous) proteins. The homologous situation clearly involves less parameters which could effect the secretion capacity of the yeast cell.

For secreted yeast proteins, like acid phosphatase or invertase, evidence has been provided that an increased gene dosage from 2-μm-based multicopy vectors leads to higher enzyme activities but does not saturate the secretory pathway [Haguenauer-Tsapis and Hinnen, 1984; Esmon et al., 1981].

The aim of this work was to put further pressure on the secretion pathway by expressing acid phosphatase under the control of a strong constitutive promoter and by choosing the defective selection marker *dLEU2* [Beggs, 1978, 1981; Erhart and Hollenberg, 1983] on a high copy number plasmid. Selection for the weakly transcribed *dLEU2* marker is likely to increase the average plasmid copy number. Concomitant with the gene dosage, the expression level of acid phosphatase is expected to go up. We have shown that this overexpression system creates a dramatic stress situation for a subpopulation of cells resulting in a defect of directed secretion along with a nongrowth phenotype and changes in the morphology. So far our report is the first to demonstrate a secretion limitation for a endogenous secretory protein in yeast. We postulate that this secretion limitation occurs late in the Golgi.

MATERIALS AND METHODS

Yeast Strains

YB18α (*MATα, his3-11, his3-15, ura3Δ5, leu2::ura3ΔSE, can*^{R}, *cir*^{0}) and the sec-mutant CJRY22-6Ba (*MATα, sec1-1, leu2-3,112, ura3-52, ade*$^-$, *sup4-3, trp1*) were used for plasmid-born acid phosphatase expression. For the construction of YB18, the strain YS18 (*leu2-3, leu2-112, his3-11, his3-15, ura3Δ5*) was used [Sengstag and Hinnen, 1988]. The sec mutant was a gift from Dr. T. Stevens [Roberts et al., 1989].

Growth Conditions

Yeast cells transformed with pDP34/GAPFL–PHO5 were grown at indicated temperatures to stationary phase in minimal medium [Sherman, 1986] supplemented with leucine (uracil selection). Cells in mid-logarithmic phase (OD_{600} of 1–2) were harvested by centrifugation (2,000*g*), washed in 0.9% NaCl and resuspended in supplemented minimal medium lacking both uracil and leucine. One-half of the cell suspension (OD_{600} = 0.4) was supplemented with uracil (leucine selection), the other half with leucine (uracil selection).

Eviction of the Genomic *LEU2* Gene, Including Its Promoter Region

Gene disruption was used to replace a *HpaI/EcoRV* fragment of the genomic *LEU2* gene [Andreadis et al., 1982] of YS18 by a *Hind*III fragment containing the *URA3* gene. In a second gene disruption step, *URA3* was replaced by a *ura3ΔSE* having a *NdeI/EcoRV* deletion.

Construction of the Expression Plasmid pDP34/GAPFL–PHO5

A 2-kbp *Bam*HI–*Pst*I fragment containing the *PHO5* gene [Bajwa et al., 1984] was used for cloning. The *PHO5* gene was cut with *Dra*I at position +4 to +9 and ligated with the synthetic oligonucleotide 5′-AATTCGATTACCAATGTTT-3′, which restored the *PHO5* sequence from position +6 to −8 and introduced a new *EcoRI* site at position −9. The GAPFL promoter is part of the promoter region of the glyceraldehyde-3-phosphate dehydrogenase gene (*GAP491 = TDH3*) [Holland and Holland, 1979, 1980] from position −198 to −27 [Janes et al., 1990]. This variant has a *Bgl*II linker at the 5′ end and a linker at the 3′ end, which restores the genuine *GAP* promoter sequence from −26 to −5 and introduces an *EcoRI* cloning site at position −4. The *Bgl*II–*EcoRI* fragment containing the GAPFL promotor variant was fused to the *EcoRI* site of the new *PHO5* gene to give the GAPFL–PHO5 expression cassette, which was cloned as a *Sal*I–*Hind*III fragment into the multicloning site of pDP34 [Hinnen et al., 1989]. At the 5′ end of the GAPFL–PHO5 cassette, the 276-bp *Sal*I–*BamHI* fragment of pBR322 is present, resulting from a subcloning step. The

plasmid was transformed into yeast strains according to the method of Klebe et al. [1983].

Acid Phosphatase Plate Assay and Enzyme Activity Tests

Acid phosphatase activity in the medium and periplasmic and cell-associated activities were determined by analyzing the culture supernatant, intact cells and toluene treated cells using an assay described by Haguenauer-Tsapis and Hinnen [1984]. Intracellular levels were calculated as difference from cell-associated and periplasmic activity. For determination of invertase activity of intact and toluene treated cells, a commercial kit (Boehringer Mannheim) was used.

Radiolabeling, Subfractionation, and Immunoprecipitation

The methods described by Riederer and Hinnen [1991] were used with slight modifications. Briefly, cells were harvested and incubated for 30 min in 400 μl minimal medium supplemented with glucose and amino acids; 100 μCi of ^{35}S-methionine was added; after 30 min, the reaction was stopped by addition of cold methionine and NaF/NaN_3. The cells were pelleted and the supernatant (=medium) was saved and bovine serum albumin (BSA) was added. The supernatant was centrifuged again in order to remove contaminating cells. For subfractionation the cell pellet was incubated in the presence of lyticase (a gift from H. Riezman, Biocenter, Basel) as described by Riederer and Hinnen [1990] but without the prior washing step. After an incubation of 1 h, the samples were centrifuged. The supernatants containing the content of the periplasmic space were saved, and BSA was added. The pelleted spheroplasts were resuspended in 200 μl 40 mM Na-citrate (pH 6.0). All following manupulations concerning immunoprecipitation, Endoglycosidase H treatment, electrophoresis and fluorography are exactly carried out as described by Riederer and Hinnen [1990]. The intensity of the bands of the fluorograms was traced by using a Shimazu densitometer.

Ludox-AM Density-Gradient Centrifugation

Cells were harvested and resuspended in H_2O (200 OD_{600}/ml); 1 ml of the cell suspensions was applied onto the top of the mixture of 5.5 ml Ludox-AM (DuPont) and 4.5-ml salt mixture [Sherman, 1991] in a 17 × 100-mm polypropylene Falcon tube. Centrifugation was for 20 min at 10°C and 22,000*g*. The gradients were fractionated (2-ml fractions) from the bottom. The three lower fractions contained denser cells. The upper two fractions contained cells that stayed on top of the gradient.

Copy Number Determination

The copy number was estimated as described by Janes et al. [1990], except that total DNA was digested by a mixture of *Sal*I, *Xho*I, and *Cla*I and that a *EcoRV–Sal*I fragment of the *LEU2* gene was used as radioactive hybridization probe. The radioactivity in the individual bands was determined using a scanner and an analyzing system (Ambis). The ratio of radioactivity in plasmid-derived band versus radioactivity in genomic band is defined as the average plasmid copy number.

Immunofluorescence and Staining of Chitin

Immunofluorescence was carried out as described by Roberts et al. [1990] using a mouse monoclonal antibody directed against acid phosphatase from yeast (kindly provided by E. Schweingruber, University of Berne, Switzerland) and a rabbit fluorescein (FITC)-conjugated anti-mouse IgG (Amersham). For double-label immunofluorescence, an affinity-purified rabbit anti yeast-actin antibody (a gift of R. Koelling, University of Dusseldorf, Germany) and a goat Texas Red conjugated IgG (Amersham) were used. Cells were analyzed in a confocal microscope (Zeiss) equipped with a laser scanner and a data-analyzing computer system. For chitin staining fixed cells were incubated with 0.1% Calcofluor white M2R (Sigma) for 10 min.

Cytochemistry and Electron Microscopy

For the preparation of samples, the procedure of van Tuinen and Riezman [1987] was used except that the polymerization of Lowicryl HM20 (Balzers Union, Liechtenstein) at 360 nm (UV-source: Philips, Type TLD, 15W/05) was done at −25°C. Sections were cut on a microtome using a diamond knife. No further contrasting was necessary for microscopy with a Siemens elmiscope type 102. Conditions for cytochemistry were according to Novick and Schekman [1979], except that DMSO was omitted and sorbitol was 0.8 M. Samples were embedded in Lowicryl HM20.

RESULTS

Acid Phosphatase Overexpression System

The yeast *PHO5* gene is regulated by its promoter in response to the concentration of inorganic phosphate in the medium [Haguenauer-Tsapis and Hinnen, 1984]. For high-level expression of acid phosphatase during optimal growth in complex media, the *PHO5* promoter was therefore replaced with the strong, constitutive GAPFL promoter, a 200-bp fragment of the yeast glyceraldehyde-3-phosphate dehydrogenase promoter [Holland and Holland, 1979, 1980].

An expression cassette comprising the GAPFL promoter, the coding region of the *PHO5* gene and the *PHO5* transcription termination signals was cloned into the 2-μm-based multicopy yeast/*E. coli* shuttle vector pDP34 [Hinnen et al., 1989], which led to expression plasmid pDP34/GAPFL–PHO5. The expression plasmid contains two yeast selection markers: the intact *URA3* gene and a defective, promoterless copy of the *LEU2* gene (*dLEU2*) [Beggs, 1978, 1981]. pDP34/GAPFL-PHO5 was used to transform YB18, a yeast strain with disrupted *ura3* and *leu2* genes.

Transformants of YB18 were pregrown under uracil selection. Cells in mid-logarithmic growth phase were collected and shifted to medium with either uracil or leucine selection. Under uracil selection YB18 cells transformed with the expression plasmid pDP34/GAPFL–PHO5 grew normally (Fig. 1). After 20 h of fermentation, approximately 70% of the acid phosphatase was secreted. About 55% thereof was retained in the periplasmic space and up to 15% reached the medium. Approximately 30% of the total acid phosphatase activity remained intracellularly (Fig. 1).

By contrast, cells shifted to leucine selection stopped to grow (Fig. 1). At 20 h of fermentation the total acid phosphatase activity per OD_{600} was approximately 2 times higher than under uracil selection. More than 50% of the acid phosphatase activity accumulated intracellularly (Fig. 1). The remaining activity was secreted to the periplasmic space, but less than 3% of the acid phosphatase reached the medium.

When invertase was concomitantly expressed using sucrose as carbon source instead of glucose at 20 h of fermentation, a growth arrest and a strong simultaneous intracellular accumulation of acid phosphatase and invertase was

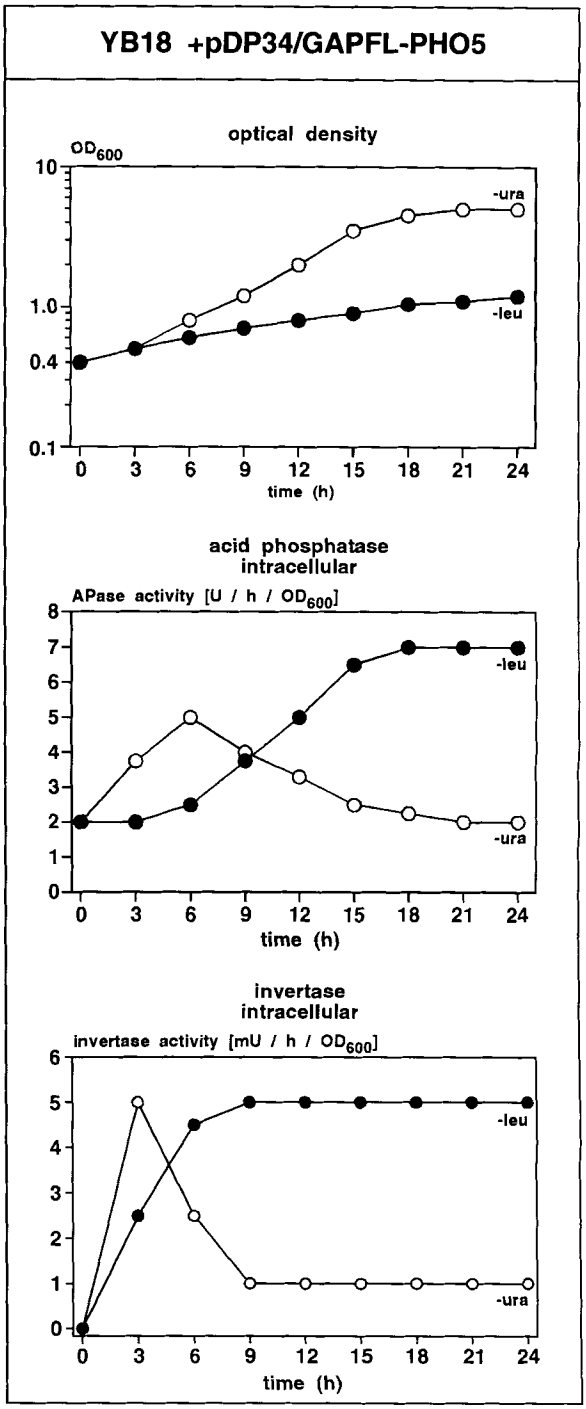

Fig. 1. Growth of YB18 transformants and intracellular acid phosphatase and invertase activity. YB18 cells transformed with pDP34/GAPFL–PHO5 were grown to logarithmic phase in minimal medium supplemented with leucine, washed, and resuspended at t = 0 in fresh medium supplemented with either uracil (-leu; closed circles) or leucine (-ura; open circles). At different time points, aliquots were assayed for optical density, and intracellular acid phosphatase and invertase activities were determined.

observed under leucine selection but not under uracil selection (Fig. 1). The concomitant accumulation of acid phosphatase and invertase under leucine selection suggests a more general effect of overexpressed acid phosphatase on secretion of secretory proteins.

It is worthwhile to state that the nongrowth phenotype under leucine selection was not caused by the selection for leucine per se, since control cells (YB18 + pDP34) grew well under leucine selection. Furthermore, in control cells hardly any acid phosphatase activity was detected indicating that the genomic *PHO5* gene remained repressed and that acid phosphatase was exclusively plasmid-born in the described system. The behavior of the cells and the cellular distribution of acid phosphatase were further analysed to better understand the basis for the phenotype observed in this overexpression system.

Distribution and Glycosylation of Newly Synthesized Acid Phosphatase

The fate of newly synthesized radiolabeled acid phosphatase was analyzed. Radiolabeled cells were separated from the culture broth and periplasmic and intracellular fractions were prepared. A transformed *sec*1 mutant strains served as control. Radiolabeled acid phosphatase from different fractions was immunoprecipitated, deglycosylated by Endoglycosidase H and separated on polyacrylamide gels. The gels were quantified by densitometric analysis of the fluorograms.

The analysis confirmed that under leucine selection acid phosphatase accumulated to a considerable degree intracellularly: up to 70% of the radiolabeled acid phosphatase stayed in the cell, roughly 30% was found in the periplasmic fraction, less than 5% was in the broth (Fig. 2, lanes 4–6). The distribution of acid phosphatase in cells under uracil selection was approximately 45% for intracellular location, approximately 40% for periplasm and up to 15% for the broth (Fig. 2, lanes 1–3). These data are in agreement with the data obtained for acid phosphatase enzymatic activity (see above).

At the permissive temperature the transformed *sec*1 mutant behaved like transformed YB18 (Fig. 2, lanes 7–12), indicating that the phenotype is independent of the strain background. At the restrictive temperature almost all of the acid phosphatase (85–95%) was located intracellularly.

The degree of glycosylation is taken as an indication for the intracellular localization of a protein: The 60-kd unglycosylated acid phosphatase is core-glycosylated to an 80-kd ER form that is subsequently converted to mature size of 120 kd during passage through the Golgi complex. YB18 cells transformed with pDP34/GAPFL–PHO5 preferentially secreted mature acid phosphatase under uracil selection conditions (Fig. 3, lane 1). Only low amounts of mature acid phosphatase were seen intracellularly (Fig. 3, lane 2). By contrast, under leucine selection nearly equal amounts of acid phosphatase with a slightly higher molecular weight (overglycosylation) were found in the periplasm (Fig. 3, lane 3) and intracellularly (Fig. 3, lane 4). Also a 80-kd core-glycosylated acid phosphatase accumulated intracellularly (Fig. 3, lane 4), but only when cells were grown for more than 10 hr under leucine selection. The transformed *sec*1 mutant grown at the permissive temperature gave essentially the same results as transformed YB18 (Fig. 3, lanes 5–8). The analysis showed that the intracellular acid phosphatase accumulated in a highly glycosylated form, typical for a glycoprotein after passage through the Golgi. Intracellular accumulation, therefore, seemed to occur in a late Golgi compartment or thereafter. Core-glycosylated acid phosphatase appeared only as a late, secondary event rather than an early accumulation.

Intracellular Localization of Acid Phosphatase and Cell Morphology

Cells with intracellular accumulation of acid phosphatase were identified by immunofluorecence. Fixed spheroplasts were stained by an anti-acid phosphatase antibody and a second antibody conjugated with a fluorescence marker. Only a minority of cells showed intense diffuse staining. Such cells were observed in cultures both under leucine and uracil selection, but were never seen in control cells (YB18 + pDP34). The immunofluoresence data suggested that acid phosphatase accumulates independent of the selection pressure only in a subpopulation of cells.

Intracellular accumulation of acid phosphatase could also lead to aberrant organelle structures as previously shown for many secretion mutants [Novick et al., 1980, 1981]. The cell

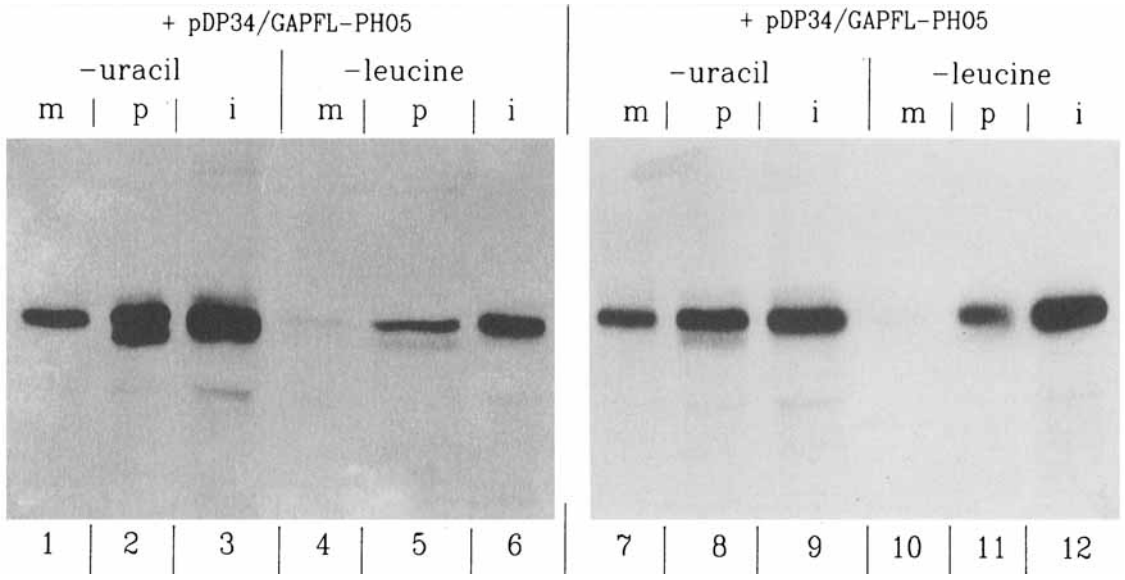

Fig. 2. Subfractionation of cell-associated acid phosphatase. YB18 and *sec1* cells transformed with pDP34/GAPFL–PHO5 were grown to logarithmic phase under uracil selection, then shifted to fresh minimal medium and incubated for 12 h under leucine (-leucine) or uracil (-uracil) selection before radiolabeling. Radiolabeled cells were spun down and the supernatant (m = medium) was collected. The cells were incubated in the presence of lyticase. After centrifugation, the supernatant (p = periplasm) was saved and the pelleted spheroplasts (i = intracellular) were resuspended in citrate buffer. Acid phosphatase was immunoprecipitated from the different subfractions (m, p, i) with a polyclonal anti-acid phosphatase antibody. The immunoprecipitates were dissolved, treated with endoglycosidase H and analysed by SDS–PAGE. A fluorogram is shown. Subfraction m (lanes 1, 4, 7, 10) represents extracellular acid phosphatase, fraction p (lanes 2, 5, 8, 11) periplasmic enzyme and fraction i (lanes 3, 6, 9, 12) intracellular acid phosphatase. The additional band observed in periplasmic subfractions (p; lanes 2, 5, 8, 11) was due to a protease contamination present in the lyticase preparation.

morphology was therefore examined under the electron microscope combined with cytochemical staining of acid phosphatase. Cells from leucine as well as uracil selection showed cellular structures that strongly resembled Golgi-derived Berkeley bodies or exaggerated ER (Fig. 4) [Novick et al., 1980, 1981]. Other structures could not be correlated with distinct organelles.

Cytochemically stained acid phosphatase was detected in ultrathin sections as electron dense material preferentially in exaggerated structures which included Berkeley bodies and ER-derived structures (Fig. 4). In control cells (YB18 + pDP34), no acid phosphatase staining occurred. Ultrastructural and cytochemical analysis showed the presence of a subpopulation of cells which accumulated acid phosphatase preferentially in the Golgi, independent of the selection conditions.

Influence of Acid Phosphatase Overexpression on Actin Pattern and Chitin Deposition

The observed phenotype with respect to aberrant morphology and deficient secretion resembled that of mutants defective in components of the cytoskeleton [Novick and Botstein, 1985; Johnston et al., 1991; Liu and Bretscher, 1992]. The actin cytoskeleton has been shown to be involved in organization of the bud region and in the directed transport of secretory vesicles to the bud [Kilmartin and Adams, 1984; Adams and Pringle, 1984; Novick and Botstein, 1985; Chant and Pringle, 1991]. During bud growth, chitin is deposited in a ring at the neck between the bud and the mother cell. The ring coincides with a ring of actin at this site which is diagnostic for bud formation and growth. Deposition of chitin at this ring can be stained by Calcofluor. Acid phosphatase and the actin pattern was analysed by double label immunofluorescence. Cells that did not stain for acid phosphatase showed actin patches throughout the cell (Fig. 5). The subpopulation of cells with intense staining of acid phosphatase showed actin patches exclusively near the surface of the cell (Fig. 5). Similarly, intense chitin staining was found all over the cell surface in a subpopulation of unbudded cells.

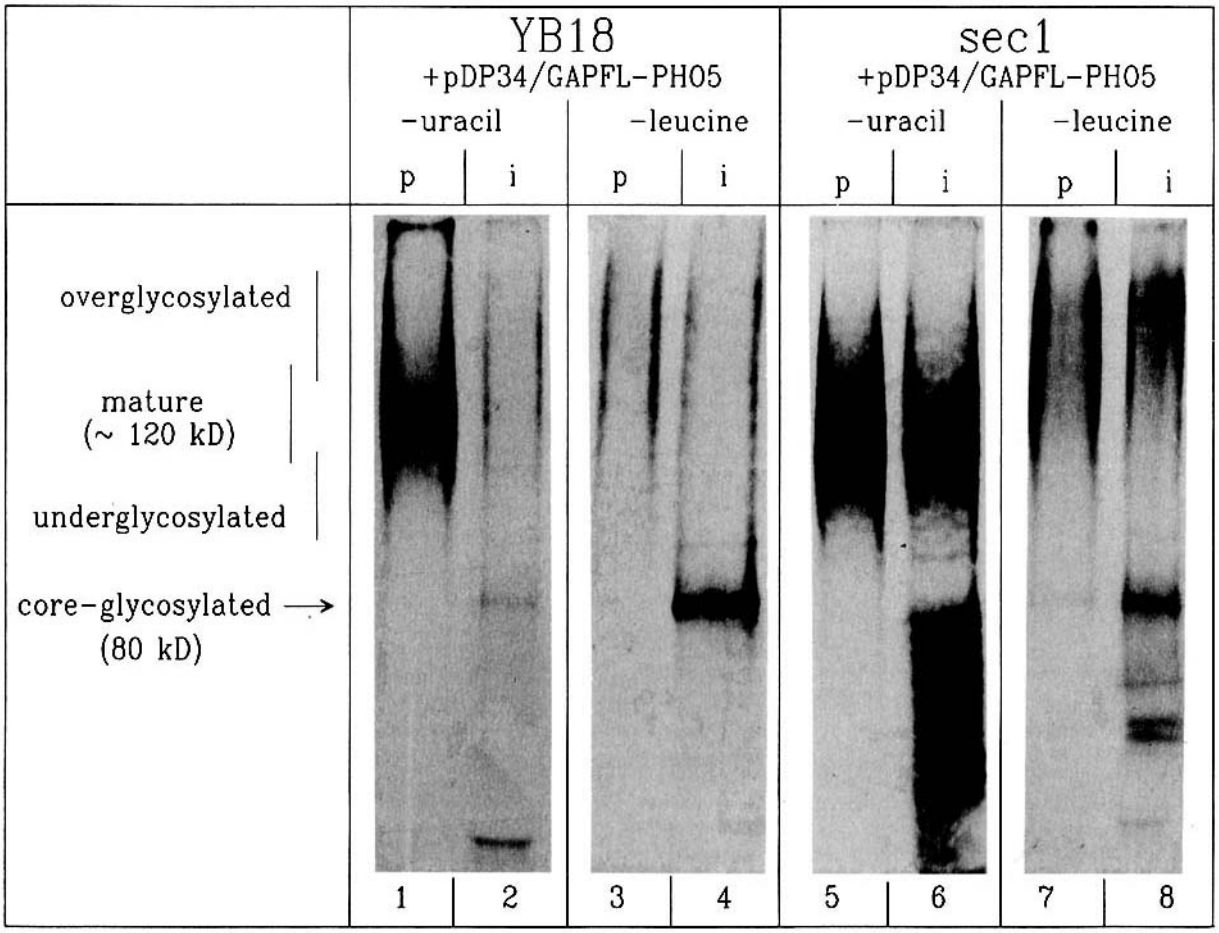

Fig. 3. Degree of glycosylation of acid phosphatase. Logarithmic YB18 and *sec1* cells transformed with pDP34/GAPFL–PHO5 were incubated for 12 h under leucine (-leucine) or uracil (-uracil) selection and then radiolabeled. The radiolabeled cells were pelleted, resuspended and further incubated with lyticase. The periplasmic (p) and spheroplast (i = intracellular) fractions were prepared by centrifugation and acid phosphatase was immunoprecipitated from the subfractions. The immunoprecipitates were analysed by SDS–PAGE. A fluorogram is shown. Highly glycosylated mature (120-kd), core-glycosylated (80-kd), underglycosylated, and overglycosylated forms of APase are indicated.

In the subpopulation of cells that overexpress acid phosphatase, the distribution of chitin and the actin pattern was changed accompanied by a disturbance of the correct formation of the bud.

Enrichment of Dense Accumulating Cells

The results so far suggested that a subpopulation of cells accumulated acid phosphatase intracellularly independent of the selection pressure. This subpopulation might have a higher plasmid copy number and an increased gene dosage for acid phosphatase which could be responsible for the growth arrest and the intracellular accumulation of acid phosphatase. We attempted to isolate and to characterize the subpopulation of accumulating cells. They were separated on the basis of their higher density on a Ludox-AM density gradient [Novick et al., 1980].

Five to ten percent of the cells carrying the pDP34/GAPFL–PHO5 expression plasmid sedimented towards the bottom of the gradient whereas control cells did not. The vast majority of dense cells from the bottom fraction of the gradient showed an intense intracellular immunofluorescent staining of acid phosphatase and an intense surface staining of chitin (Fig. 6), an indication for acid phosphatase accumulation and a defect in bud formation and growth. Indeed, only 20% of the cells were able to form buds and to grow on rich medium plates. Actin patches exclusively at the cell surface also went along with this nongrowth phenotype.

Radiolabeling of dense cells showed that the total amount of acid phosphatase was only slightly lower under leucine selection compared to uracil selection suggesting that the dense

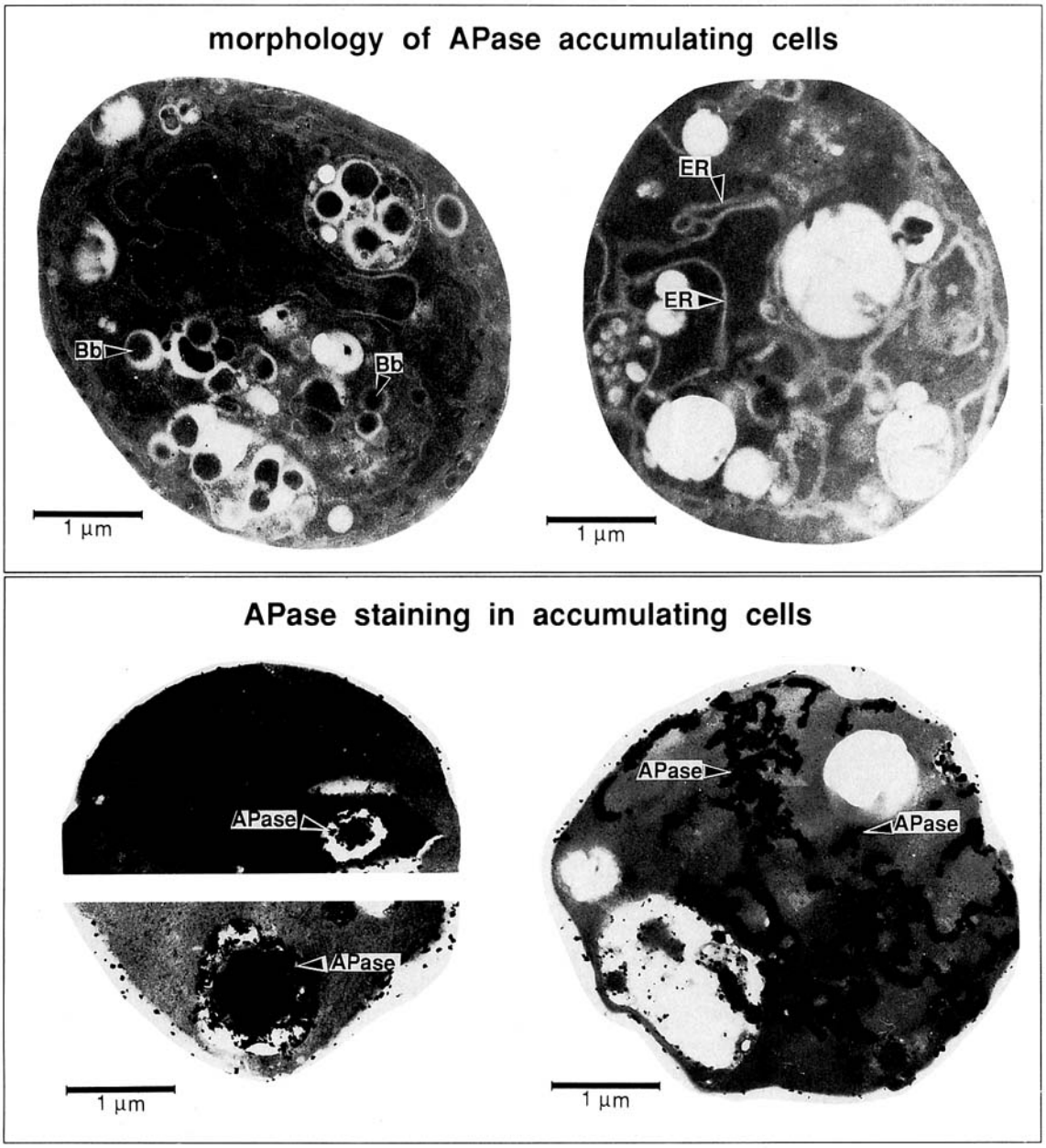

Fig. 4. Morphology of transformed YB18 cells and ultrastructural localization of cell-associated acid phosphatase. Logarithmic YB18 cells transformed with pDP34/GAPFL–PHO5 were exposed to leucine or uracil selection. After 12 h cells were fixed, dehydrated, embedded, and processed for electron microscopy. For substructural localization an acid phosphatase reaction was carried out upon fixation of cells. Afterward, the samples were postfixed, dehydrated, embedded and analysed in an electron microscope. Ultrathin sections of cells with typical exaggerated structures are shown. Bb, Berkeley bodies; ER, exaggerated endoplasmic reticulum; APase; electron-dense staining of acid phosphatase.

cells do not starve on leucine. In addition, the subsequent incubation of dense cells under either uracil or leucine selection led in both cases to the same pattern of subcellular localization of newly synthesized, radiolabeled acid phosphatase: up to 70% accumulated intracellularly, about 30% reached the periplasm with less than 5% in the broth (Fig. 7, lanes 7–12). Most of the acid phosphatase was overglycosylated. These features were exactly the same as initially seen for a cell culture under leucine selection. The majority of cells in the top fraction of the gradient behaved like normal cells unaffected by acid phosphatase expression. They showed only a faint immunofluorescent signal (Fig. 6), normal actin pattern, and normal chitin deposition at

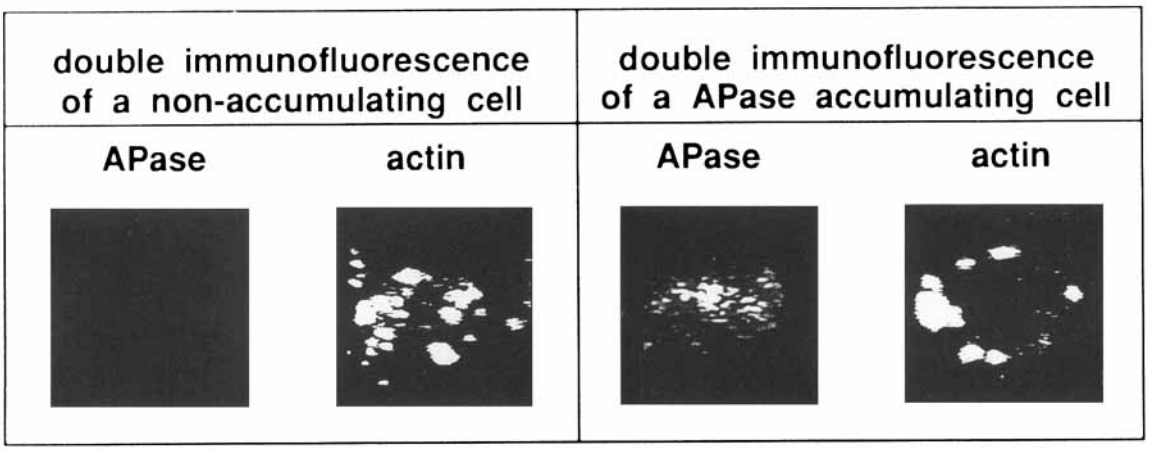

Fig. 5. Actin pattern. YB18 cells bearing pDP34/GAPFL–PHO5 were incubated under leucine or uracil selection for 12 h. A double-label immunofluorescence was carried out using an anti-actin and anti-acid phosphatase antibody. Actin (actin) and acid phosphatase (APase) were stained by a Texas Red-conjugated and an FITC-conjugated second antibody, respectively. A typical nonaccumulating cell and an APase accumulating cell is presented. Digitalized images are shown.

the neck of the bud (Fig. 6), which is characteristic for unaffected, directed secretion, normal bud formation and growth. Indeed, 75–80% of the cells were able to grow on rich medium plates.

The total radiolabeled acid phosphatase under leucine selection was only 10–20% of the amount determined under uracil selection suggesting that cells of low density starve on leucine. A considerable part of the newly synthesized radiolabeled acid phosphatase was found in the periplasmic fraction (about 45%; Fig. 7, lane 2) and in the broth (about 15%; Fig. 7, lane 1). Approximately 40% was located intracellularly (Fig. 7, lane 3). Only acid phosphatase of mature size was detected. Under leucine selection (Fig. 7, lane 4–6) results resemble those for dense cells of the bottom fraction.

The total acid phosphatase activity per cell is consistently different in the subpopulations. Cells from the bottom fraction showed an approximately twofold higher total acid phosphatase activity per cell compared to cells from the top fraction and accumulated higher amounts of acid phosphatase intracellularly (about 60% versus about 30% of total cell-associated acid phosphatase activity).

The higher level of synthesis correlated well with an approximately two fold higher average plasmid copy number: The average copy number of cells derived from bottom fractions was determined to be 18, whereas the average copy number in cells from the top fractions was 8. The higher plasmid copy number increases the gene dosage for acid phosphatase and could trigger the effects on growth and secretion.

In summary, these results demonstrate that cells with a high density have a low viability and synthesize twofold more acid phosphatase in response to a higher gene dosage resulting from the twofold higher plasmid copy number. These cells show a pronounced accumulation of overglycosylated acid phosphatase associated with an aberrant organization of the actin cytoskeleton and coincide with a defect in bud formation and growth. By contrast, cells with a low density are able to grow under uracil selection but show a low viability and a reduced synthesis of acid phosphatase under leucine selection.

DISCUSSION

Saccharomyces cerevisiae has been widely used for the expression of heterologous proteins [Moir, 1989; Hinnen et al., 1989]. The capacity of the secretory pathway seems, however, to be rather variable for different heterologous proteins. The question still remains whether the pathway has its limitations, particularly in cells where secreted proteins are overexpressed. We used a genuine yeast protein, acid phosphatase, which is secreted to the periplasmic space, to evaluate the overall performance of the secretory pathway.

To establish an overexpression situation, acid phosphatase was constitutively expressed under the control of a strong promoter placed on a 2-μm-based high copy number plasmid pDP34 with an *URA3* and *dLEU2* selection marker.

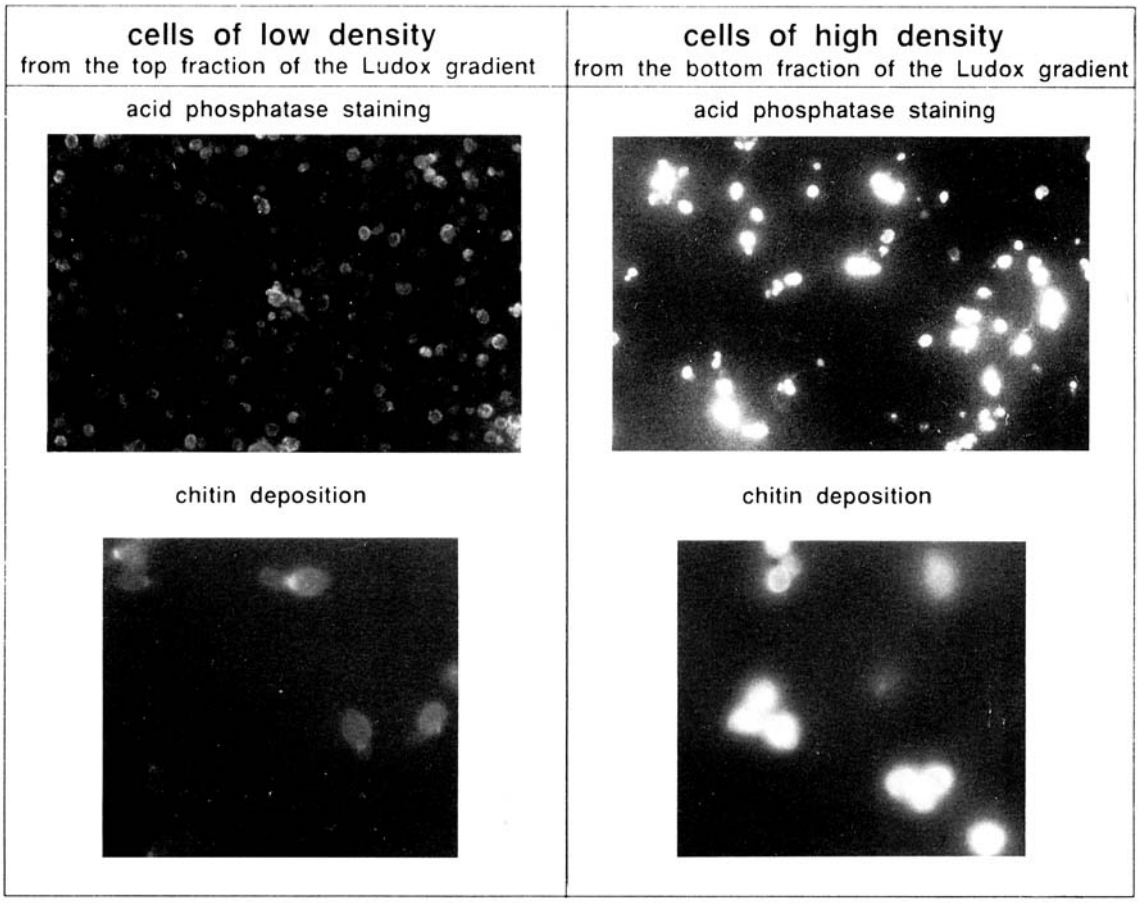

Fig. 6. Separation of nonaccumulating cells from dense accumulating cells by Ludox-AM density-gradient centrifugation. YB18 cells transformed with pDP34/GAPFL–PHO5 were exposed to uracil or leucine selection. After 6 h, cells were harvested, resuspended in water, and applied to a 55% Ludox-AM solution. After centrifugation, the gradients were fractionated from the bottom. Cells in the bottom and top fractions of the gradient were analysed for acid phosphatase accumulation and chitin deposition. Intracellularly accumulating acid phosphatase was stained by immunoflourescence using an anti-acid phosphatase antibody in conjunction with an FITC-conjugated second antibody. Chitin was stained by Calcofluor white M2R.

Partitioning of 2-μm plasmids is known to be rather inefficient [Futcher, 1983, 1988], leading to a broad distribution of copy number per individual cell. This explains the presence of subpopulations of cells with low and high copy numbers.

Under uracil selection, all transformed cells, including those with low copy number, can complement the uracil auxotrophy of the host strain via plasmid-born expression of the *URA3* marker. Complementation of the leucine auxotrophy, however, requires a higher copy number [Erhart and Hollenberg, 1983], since the expression level of the *dLEU2* gene is low due to its deleted promoter [Janes et al., 1990].

The plasmid copy number also determines the gene dosage for the expression of acid phosphatase, which is likely to have a feedback on the balance between complementing gene product, growth, copy number, and acid phosphatase expression [Janes et al., 1990]. Under leucine selection, we see two overlapping effects that lead to the nongrowth phenotype of the culture: leucine starvation for one subpopulation of cells with low copy number, and growth arrest for another subpopulation of cells with a high copy number accumulating acid phosphatase. A subpopulation of dense cells with excess acid phosphatase is also present under uracil selection but is hidden by most normally growing cells. Therefore the phenotype of normal growth and secretion of a culture under uracil selection reflects the behavior of cells with a low copy number.

Analysis of the phenotype revealed that only a subpopulation of transformed cells showed severe effects on their morphology and physiology.

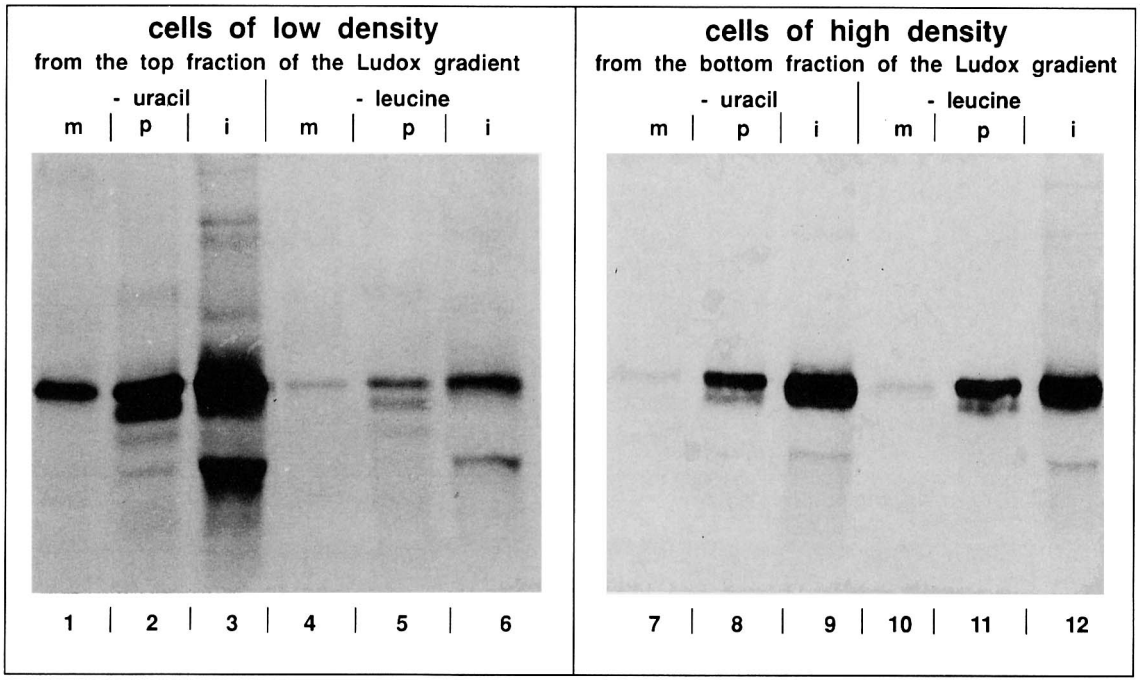

Fig. 7. Rabiolabeling and subfractionation of cells deriving from different fractions of a Ludox-AM density gradient. YB18 cells transformed with pDP34/GAPFL–PHO5 were exposed to uracil or leucine selection. After 6 h, cells were harvested, resuspended in water and applied to a 55% Ludox-AM solution. After centrifugation, the gradients were fractionated from the bottom. Cells of low density from the top fraction and cells of high density from the bottom fraction were washed and radiolabeled either in the absence of leucine (-leucine) or uracil (-uracil). The cells were spun down and the cell-free culture supernatant (m) was collected. Periplasmic (p) and spheroplast (i = intracellular) fractions were prepared by centrifugation after treating the cells with lyticase. Acid phospatase of the medium, of the periplasmic space (p) and of intracellular compartments (i) was immunoprecipited, treated with endoglycosidase H, and analysed by SDS–PAGE. A fluorogram is shown.

The intracellular accumulation of acid phosphatase allowed the separation of these cells on the basis of their greater density.

Although dense cells from the bottom fraction of the gradient do not starve on leucine they are in an irreversible physiological state which does not allow bud formation and directed secretion to the bud. This phenotype correlates with an approximately twofold higher average plasmid copy number and rate of synthesis of acid phosphatase. Rather, the intracellular accumulation of continuously expressed acid phosphatase results from a saturation of the secretion pathway or from a rate limitation, but it seems to be just due to a shutdown of the secretion machinery reflected by the severe effects on secretion, growth, and morphology.

By contrast, cells with a low density from the top of the gradient can grow and secrete acid phosphatase under uracil selection, but they starve under leucine selection. The results showed that already a approximately twofold difference in gene dosage for acid phosphatase had a dramatic effect on secretion and growth. Obviously, secretion and growth is a well-balanced system that has the ability to take a certain overexpression stress but seems to collapse at a certain point when the level of expression is too high. From this we must conclude, that the particular subpopulation of cells with an approximately two-fold higher plasmid copy number already are at this point and have reached the limit of secretion capacity for acid phosphatase.

Intracellularly accumulated acid phosphatase was in a highly glycosylated form which is typical for a Golgi location. Overglycosylation may be the result of long exposure to Golgi glycosyltransferases and reflects kinetic differences in transport. Most of the accumulated acid phosphatase was located in aberrant cellular structures. They were seen in the electron microscope in numerous cells and often resemble Berkeley bodies, which are derived from Golgi structures [Novick et al., 1980, 1981]. Berkeley bodies were also observed in a number of yeast mutants late in the secretory pathway (*sec14*) [Novick et al., 1980], *sec7* [Novick et al., 1980],

myo2 [Johnston et al., 1991], *ypt1* [Segev et al., 1988], *act1* [Novick and Botstein, 1985; Novick et al., 1989].

Vectorial transport is normally directed to the growing bud region of the cell [Field and Schekman, 1980; Novick and Schekman, 1979; see for review: Drubin, 1991; Chant and Pringle, 1991], but this route appears to be blocked in the dense cells filled with acid phosphatase. The intracellular accumulation of acid phosphatase and simultaneously of invertase suggests a general block of vectorial secretion, which is best documented by the growth arrest. Nevertheless, there is a considerable amount of overglycosylated acid phosphatase released to the periplasmic space, which may be via a slower course of undirected diffusion of transport vesicles to the cell surface. This route is inhibited in *sec1* mutants, indicating that the post-Golgi-acting *SEC1* gene product is involved in the fusion of these vesicles with the plasma membrane. Along these lines the aberrant chitin deposition at the plasma membrane can well be due to mislocation of chitin synthetase to the cell surface by undirected transport.

The phenotype of dense cells has its parallel in actin (*act1-1*) [Novick and Botstein, 1985], myosin (*myo2–6*) [Johnston et al., 1991] and tropomyosin (*tpm1*) [Liu and Bretscher, 1992] mutants. The disrupted cytoskeleton prevents transport to the bud and leads to an accumulation of vesicles and to an abnormally large pool of secretory proteins inside the cell. The defect in efficient vectorial transport of secretory vesicles along actin cables may then liberate these vesicles to follow undirected diffusion to the cell surface. In contrast to the *act1-1, myo2-6,* and *tpm1* mutant in cells accumulating acid phosphatase due to overexpression secretory vesicles were not observed. We therefore assume that the formation of secretory vesicles and/or interaction of budding vesicles with the actin cytoskeleton is the limiting step in the export of acid phosphatase.

The nongrowth phenotype following acid phosphatase overexpression is a useful tool with which to isolate suppressor mutants and to analyze cellular stress situations.

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