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Down-regulation of Model Yeast Proteins by Ubiquitin-dependent Proteolysis

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Summary

Ubiquitination is a versatile tool used by all eukaryotic organisms for controlling the stability, function, and intracellular localization of a multitude of proteins. I will attempt to bring together our recent data on the down-regulation of two yeast model proteins, the galactose transporter Gal2 and fructose-1,6-bisphosphatase, by ubiquitin-dependent proteolysis triggered by the addition of easily fermentable carbon sources.

Key words

Yeast • Ubiquitin-dependent proteolysis • Vacuole • Proteasome

Introduction

There is now general agreement that the posttranslational conjugation of ubiquitin to lysine residues of a set of naturally and conditionally unstable proteins serves as the primary signal of their instability. Ubiquitin is a 76-amino-acid polypeptide, whose conjugation to proteins involves the sequential transfer of the ubiquitin moiety to substrate proteins through the E1-E2-E3, i.e. ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-ligase (E3) thiol ester cascade culminating in the formation of an amide (isopeptide) bond between the C-terminal glycine Gly-76 of ubiquitin and the ε-amino group of specific lysine residues within the substrate protein (Weissman 2001, Pickart 2004). Monoubiquitinated proteins are often (but not always) further modified by the attachment of additional ubiquitin molecules giving rise to polyubiquitin chains. Since ubiquitin is a long-lived protein in wild-type cells, it can be recycled from ubiquitin-protein conjugates by the action of specific thiol proteases named deubiquitination enzymes or isopeptidases, and thereby reused in multiple rounds of the ubiquitin cycle.

Attachment of ubiquitin to substrate proteins has distinct mechanistic roles in two different intracellular proteolytic pathways. The best known role for the linkage of ubiquitin is to mark cytosolic and nuclear proteins that are subjected to hydrolytic degradation by the 26S proteasome. For most of the proteasome substrates, the attachment of a polyubiquitin chain in which four ubiquitin units are linked by isopeptide bond between Lys-48 of ubiquitin and the C-terminal group of the following ubiquitin are found. However, ubiquitination of a multitude of cell-surface nutrient and ion transporters or signal-transducing receptors signals their internalization into the endocytotic pathway(s) and subsequent proteolysis in the vacuole.

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Importantly, in all known cases of yeast plasma membrane proteins, a single ubiquitin moiety or di- to tri-ubiquitin chains, in which ubiquitin molecules may be linked through Lys-63, appear to suffice to trigger their internalization into the interior of the cell (Horák 2003).

Like other eukaryotic organisms, yeasts capable of utilizing several different sugars as well as some non-sugar carbon sources selectively ferment glucose even when alternative carbon and energy sources (galactose, maltose, ethanol, etc.) are simultaneously available. This is achieved by a complex regulatory network which operates at two different levels, at the level of transcription and/or at the posttranslational level. At the posttranslational level, glucose induces a rapid loss of activity of gluconeogenetic enzymes and of several sugar transporters and stimulates an increase in the rate of degradation of these proteins by a process known as catabolite inactivation or catabolite degradation (Horák 1997).

This short review is confined largely to our studies of the mechanism of selective, ubiquitin-dependent proteolytic degradation of two yeast model proteins in response to glucose, namely, the Gal2 transporter and the fructose-1,6-bisphosphatase, representing substrates of vacuolar and proteasomal proteolysis systems, in response to glucose.

Ubiquitination machinery and types of ubiquitin chains

In the yeast *Saccharomyces cerevisiae*, galactose transport across the plasma membrane is mediated by the Gal2 transporter (Gal2p) predicted to possess 12 membrane-spanning helices that is inducible by its own substrate. Our initial kinetic studies with the Gal2p have revealed that its capacity to mediate galactose transport is rapidly and irreversibly reduced when *S. cerevisiae* cells grown on galactose are transferred to a glucose medium containing cycloheximide, an inhibitor of cytosolic protein synthesis (Horák and Wolf 1997). Characteristics of this process implies that catabolite inactivation of Gal2p could be due to its breakdown. Indeed, using polyclonal antibody raised against the N-terminal part of Gal2p we have demonstrated (Horák and Wolf 1997) that Gal2p inactivation is caused by its hydrolysis in response to glucose with a half-time of about 1 h. Moreover, Gal2p apparently enters the endocytotic pathway(s) and is degraded mainly in the vacuole, as demonstrated by its stabilization in two mutants conditionally defective in the internalization step of endocytosis (*end3* and *end4* mutants) and mutants defective in proteinases yscA and yscB, which play a central role in the general proteolytic function of the vacuole (*pra1* and *prb1* mutants). The vacuolar proteolysis of Gal2p was also consistent with its accumulation in the vacuole observed by subcellular fractionation and indirect immunofluorescence. In contrast, Gal2p appears to be proteasome-independent since its half-time is unaffected in mutants defective in both the regulatory (*cim3*, *cim5*) and catalytic (*pre1*, *pre2*) subunits of the 26S proteasome. Finally, Gal2p appears to be ubiquitinated in response to glucose addition; a ladder of Gal2-ubiquitin conjugates over the entire molecular mass spectrum was detected. Taken together, the above results demonstrate that glucose-induced proteolysis of Gal2p is dependent on endocytosis and vacuolar proteolysis and that involvement of ubiquitin in this process is highly probable.

To show that ubiquitination of the Gal2p is actually the prerequisite for posttranslational regulation of its activity by glucose-triggered proteolysis, the fate of Gal2p was examined in yeast strains carrying mutations that inactivate (*ubc* mutations) or at least strongly reduce (*rsp5* mutation) activity of some components of the ubiquitin-conjugating pathway (Horák and Wolf 2001). Our immunoblot analysis of the Gal2p fate demonstrated that only Ubc1p, Ubc4p, and Ubc5p, members of the functionally redundant family of ubiquitin-conjugation enzymes (E2´s; eleven in total) and Rsp5p ubiquitin-ligase (E3) are necessary and sufficient for Gal2p ubiquitination at the cell surface, internalization by endocytosis, and degradation in the vacuole. Consistent with this view is also our observation that a loss of the free intracellular pool of ubiquitin due to a gene mutation of *DOA4* (the gene encoding Doa4p, one of the deubiquitination enzymes) severely impairs glucose-triggered Gal2p degradation and that this defect can be suppressed by the overexpression of ubiquitin (Horák and Wolf 2001).

Although Gal2p displays a set of a high-mol-mass ubiquitin conjugates formed in response to glucose, it obviously escapes recognition and subsequent proteolysis by the proteasome (Horák and Wolf 1997). To find an explanation for this apparent paradox, we tried to elucidate whether the observed ubiquitination patterns result from Gal2p modifications by ubiquitin chains of different length or from the addition of single ubiquitin molecules to multiple lysine residues (Horák and Wolf 2001). To decide between these alternatives, we

examined the fate of Gal2p in *doa4* mutant cells overexpressing either wild-type or mutant ubiquitins incompetent to form ubiquitin chains *via* K29, K48, K63, and all other lysines, respectively. Overexpression of any of the ubiquitin mutant forms described above restored proteolysis of the Gal2p as well as its internalization in *doa4* mutant cells to the same extent as overexpression of wild-type ubiquitin. Altogether, these results indicate that monoubiquitination of Gal2p at several lysines through the Ubc1p-Ubc4p-Ubc5p triad of E2´s and Rsp5p is sufficient to signal internalization of the protein into the endocytotic pathway.

Although inactivation conditions and the ultimate fate of the fructose-1,6-bisphosphatase (FBPase) are similar to the Gal2p, modification of FBPase by polyubiquitin chain-containing isopeptide linkages at Lys-48 of ubiquitin is prerequisite for degradation by the cytosolic proteasome (Schork *et al*. 1995, Hammerle *et al*. 1998, Schule *et al*. 2000).

The role of glucose-sensing/signaling pathways in Gal2p and FBPase degradation

To identify the mechanism(s) that determine the different fates of Gal2p and FBPase observed during their catabolite inactivation, i.e., proteasomal versus vacuolar degradation, we tried first to elucidate whether these differences could be due to utilization of distinct glucose sensing/signaling pathways by different proteolytic systems. For glucose signaling in yeast, at least three signal transduction pathways exist, and the ultimate response is a change in synthesis or degradation of specific sets of proteins. Therefore, we started our analysis with a search for the potential functions of the main protein components of glucose sensing/signaling systems in the Gal2p and FBPase degradation. Rather surprisingly, we found that two distinct proteolytic systems responsible for Gal2p and FBPase degradation utilize most (if not all) protein components of the same glucose signaling pathway (Horák *et al*. 2002). Indeed, initiation of Gal2p and FBPase degradation appears to require rapid transport of glucose or those glucose-related compounds that are substrates of Hxt transporters and that are at least partly metabolized (e.g. phosphorylated) by hexokinase Hxk2p. The signal generated in this way is subsequently transduced through Reg1p and Grr1p, two downstream components of the glucose signaling pathway. How these proteins contribute to Gal2p and FBPase degradation is not clear at present. Nevertheless, Grr1p is one of variable F-box proteins that account for specificity of several different SCF complexes with an E3 ubiquitin-ligase activity. Most of the SCF complexes are comprised of a Ring-type protein Rbx1p, a cullin Cdc53p, and an adaptor protein Skp1p and are tightly associated with a particular E2, Cdc34p. These data prompted us to examine the fates of Gal2p and FBPase in mutants defective in individual subunits of the SCF complex. Using immunoblot analysis we observed a very strong stabilization of both the Gal2p and FBPase in *cdc34*, and *skp1* mutant strains and, in addition, Gal2p and FBPase were stabilized specifically in *rbx1* and *cdc53* mutant cells, respectively. The significance of only partial overlap in the SCF composition remains to be elucidated. Another protein identified to be involved in the transduction of the signal that regulates catabolite inactivation of Gal2p and FBPase is Reg1p, a regulatory subunit of the PP1 protein phosphatase complex. In this connection, we have shown that Reg1p participates in cooperation with Bmh1p and Bmh2p, two members of the 14-3-3 protein family, in the regulation of the glucose-induced degradation of maltose transporter Mal61, but not of Gal2p and FBPase (Mayordomo *et al*. 2003)

Search for new catalytic and regulatory components of the ubiquitination machinery

To obtain a global view of all genes involved in FBPase degradation, we performed a genome-wide screen for additional genes involved in this process (Regelmann *et. al*. 2003). The approach used consisted in screening a collection of about 5000 homozygous diploid strains *S. cerevisiae* each lacking a nonessential gene. Our studies led to the identification of eight novel *GID* genes required for proteasome-dependent catabolite degradation of FBPase. Although no function for most of the *GID* gene products has yet been suggested, at least most of them appear to be a part of a soluble protein complex of about 600 kDa. Further studies attempting to determine the precise molecular composition, the topology, the dynamics and the function of the putative complex are in progress. Remarkably, none of the *GID* gene products is involved in glucose-induced Gal2p degradation.

For a near future we plan to use a similar reverse genomic approach in the identification of novel genes, whose products participate in the vacuolar proteolysis of Gal2p. Since Gal2p proteolysis is not essential for

cellular life under the conditions of catabolite inactivation, genes exclusively affecting this process are not expected to be essential for viability. Attempts to elucidate the molecular composition, cell localization, nature of mutual interactions, and functions of all newly discovered gene products will follow their identification.

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