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Redundant targeting of Isr1 by two CDKs in mitotic cells.

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Abstract

Protein phosphorylation is an essential regulatory mechanism that controls most cellular processes, integrating a variety of environmental signals to drive cellular growth. Isr1 is a negative regulator of the hexosamine biosynthesis pathway (HBP), which produces UDP-GlcNAc, an essential carbohydrate that is the building block of N-glycosylation, GPI anchors and chitin. Isr1 was recently shown to be regulated by phosphorylation by the nutrient-responsive CDK kinase Pho85, allowing it to be targeted for degradation by the SCF^{CDC4}. Here, we show that while deletion of *PHO85* stabilizes Isr1 in asynchronous cells, Isr1 is still unstable in mitotically arrested cells in a *pho85*Δ strain. We provide evidence to suggest that this is through phosphorylation by CDK1. Redundant targeting of Isr1 by two distinct kinases may allow for tight regulation of the HBP in response to different cellular signals.

Introduction

In all eukaryotes, cell cycle regulation is driven by a set of cyclin-dependent kinases that contain a catalytic subunit (a CDK) and a cyclin. One way in which this cyclin CDK complex is regulated is through the periodic destruction of the cyclin subunit. The budding yeast *Saccharomyces cerevisiae* encodes a single essential CDK whose catalytic subunit, called Cdk1 (or Cdc28), associates with any of nine cyclins (Morgan, D.O. 2007). While this is the only CDK required for cell cycle regulation, another CDK, called Pho85 also promotes cell cycle progression and associates with cell cycle regulated cyclins, known as Pcls (Carroll et al. 2001; Huang et al. 2007). One such example is Pcl1, which is active in G1 (Espinoza et al. 1994). In addition to cell-cycle regulated Pcls, Pho85 can also associated with several Pcls regulated by changes in nutrients, such as glycogen levels (Huang et al. 1998). Both Cdk1 and Pho85 utilize a proline directed consensus sites (S/T-P) (O'Neill et al. 1996; Holt et al. 2009).

One well-characterized function of *CDK1* is targeting substrates for degradation by the SCF. The SCF is a ubiquitin ligase composed of several core subunits, including a cullin and RING subunit, and any of several specificity subunits called F box proteins (Willems et al. 2004). Two of these F box proteins, Cdc4 and Grr1, have been shown to specifically recognize proteins once they have been phosphorylated (Skowyra et al. 1997; Lyons et al. 2013). While these F box proteins do target proteins after phosphorylation by other kinases, such as Snf1 in the case of Grr1 (Benanti et al. 2007), most Cdc4 and Grr1 substrates are recognized only after phosphorylation by CDK. In the case of at least one Cdc4 substrate, Sic1, this targeting is carried out by both Pho85 and Cdk1 (Nishizawa et al. 1998).

Previously, we identified Inhibitor of Staurosporine Resistance 1 (Isr1) as a Cdc4 substrate targeted by Pho85 (Mark et al. 2014; Alme et al. 2020). Isr1 is a poorly characterized putative kinase previously been thought to be involved in cell wall homeostasis (Miyahara et al. 1998; Mehlgarten et al. 2007). Recently, we discovered that Isr1 is indirectly involved in cell wall homeostasis via inhibition of the hexosamine biosynthesis pathway (HBP) (Alme et al. 2020), which produces Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) from glucose and glutamate (Milewski et al. 2006). UDP-GlcNAc is the precursor to three molecules: chitin, GPI anchors, and N-glycosylation (the last two of which are conserved to humans). Chitin is a minor, but critical structural component of the yeast cell wall and GPI anchors are the main protein component (Orlean 2012). When modestly overexpressed, *ISR1* caused resistance to calcofluor white, a cell wall agonist that binds chitin (Mehlgarten et al. 2007; Alme et al. 2020) and sensitivity to tunicamycin (Alme et al. 2020), an ER stress agent and N-glycosylation inhibitor (Yoo et al. 2018). Both of these phenotypes are consistent with a reduction in UDP-GlcNAc synthesis. Chitin production is directly proportional to UDP-GlcNAc synthesis (Lagorce et al. 2002) and a shortage of UDP-GlcNAc would exacerbate the reduction in N-glycosylation caused by tunicamycin. At high levels of overexpression, *ISR1* was lethal to the cell, suggesting it inhibits an essential pathway. All of these phenotypes were rescued by overexpression of the first enzyme in the hexosamine biosynthesis pathway, glutamine: fructose-6-phosphate aminotransferase (GFA1) or addition of exogenous glucosamine, which by-passes GFA1 (Zheng et al. 2000; Alme et al. 2020). GFA1 is phosphorylated in an Isr1-dependent manner and mutation of these phosphorylation sites on Gfa1 ameliorates lethality caused by *ISR1* overexpression. Based on these phenotypes, we proposed the following model: Isr1 phosphorylates and inhibits Gfa1, and in doing so inhibits the hexosamine biosynthesis pathway (Alme et al. 2020). This, in turn, reduces both cell wall biosynthesis and the production of cell surface proteins.

Isr1 is targeted for degradation by Pho85

Given its role in inhibiting production of UDP-GlcNAc, an essential carbohydrate, tight regulation of Isr1 activity is critical to cellular homeostasis. Isr1 is a very low abundant protein and is highly unstable, with a half-life of approximately 30 minutes (Ghaemmaghami et al. 2003; Alme et al. 2020). Transcriptional data suggested that Isr1 levels alternate in the cell cycle (Spellman et al. 1998). Similarly, we find that Isr1 protein levels vary at different cell cycle stages, peaking at G1/S at the time of budding and largely absent during mitosis (Fig 1A). For as yet unclear reasons, alpha factor arrest reduces Isr1 levels more than other G1 arrest methods (unpublished data). Previously, Isr1 had been identified as an *in vitro* substrate of Pho85-Pcl1, which functions in G1 progression (Dephoure et al. 2005). Previously, we deleted *PHO85* and observed that this stabilized Isr1 protein levels, suggesting that phosphorylation by Pho85 targets Isr1 for degradation (Alme et al. 2020). Low-phosphate media, which inhibits Pho85 activity (Schneider et al. 1994), also stabilized Isr1 (Alme et al. 2020). Deletion of *PCL1* resulted in only partial stabilization of Isr1 compared to deletion of *PHO85* (Alme et al. 2020), suggesting other Pcls also contribute to Isr1 phosphorylation.

Isr1 appears to be a substrate of the SCF^{Cdc4} complex. Cdc4 identifies its substrates by binding phosphodegrons with the optimal sequence (S/T)-P-X_{2-4} -(S/T) in which both (S/T) are phosphorylated, often by CDK kinases (Tang et al. 2005; Hao et al. 2007; Edenberg et al. 2014). Isr1 contains two putative Cdc4 phosphodegrons and mutation of these phosphosites (*ISR1-PD*) completely stabilized Isr1 protein levels (Alme et al. 2020). Importantly, some cell cycle dependent variation in the levels of Isr1 persists even in the *ISR1-PD* strain, suggesting that its cell cycle regulation may be both transcriptional and post-transcriptional (Fig 1A). These data suggest that Pho85 phosphorylates Isr1 in response to nutrient and/or cell cycle signals, allowing SCF^{Cdc4} to then target it for degradation (Fig 2).

In contrast to these findings, a previous study had suggested that Pho85 might activate Isr1 (Mehlgarten et al. 2007) . Mehlgarten et al. found that Isr1 is a high copy suppressor of the *Kluyveromyces lactis* protein toxin zymocin and deletion of *PHO85* restored zymocin sensitivity in the context of *ISR1* overexpression. The authors suggested that the loss of zymocin suppression upon *PHO85* deletion indicates that Pho85 activates Isr1. However, our data suggest that the zymocin suppressor phenotype of high copy *ISR1* is likely due to a reduction in chitin levels in these cells. As chitin is the cellular receptor for zymocin (Jablonowski et al. 2001) , *ISR1* overexpression would render the cells less sensitive to the toxin. We believe *PHO85* deletion restores zymocin sensitivity due to the pleotropic effects of *PHO85* deletion on cell wall homeostasis. This also explains why deletion of *PCL1* did not alter zymocin suppression by high copy *ISR1*, as it does not appear solely responsible for Isr1 stabilization.

CDK phosphorylates Isr1 during mitosis

While deletion of *PHO85* at steady state stabilizes Isr1 protein levels comparably to the phosphodegron mutant of Isr1, Isr1-PD (Alme et al 2020), this does not hold true when cells are arrested in mitosis. When cells are arrested with nocodazole, Isr1-PD remains completely stable (Fig. 1B), but deletion of *PHO85* results in only partial stabilization of Isr1 (Fig. 1C). This suggests that other kinases are capable of targeting Isr1 as well. As the phosphosites mutated in the Isr1-PD allele are CDK consensus sites, we tested if CDK might target Isr1 under these conditions. We utilized the *cdk-as1* allele, which renders the kinase sensitive to the inhibitor 1-NM-PP1 (Bishop et al. 2000), and found that this resulted in a partial stabilization of Isr1 compared to wildtype in nocodazole arrested cells (Fig. 1D). This suggests that Cdc28 is also capable of targeting Isr1 for degradation. Given that Isr1 transcription appears largely confined to the time of budding, it remains possible that CDK activity towards Isr1 is not relevant in a normal cycling cell. However, when cells are arrested in mitosis due to activation of either the spindle checkpoint or the DNA damage checkpoint, this secondary phosphorylation by Cdk1 may be critical to down-regulate Gfa1 during the arrest.

Perspective

In contrast to Isr1, which is very low abundant, GFA1 is extremely abundant and stable throughout the cell cycle (Ghaemmaghami et al. 2003), although levels increase in response to alpha factor in an Isr1-independent fashion (Zheng et al. 2000; Alme et al. 2020).

Regulating Isr1 may be a less energetically costly way of controlling UDP-GlcNAc production than regulating enzymes of the HBP directly, since these phosphorylations can be readily removed and inhibition can be relieved without having to resynthesize a highly abundant protein such as Gfa1. Activity of the hexosamine biosynthesis pathway is directly proportional to chitin production and chitin production therefore fluctuates with HBP activity (Lagorce et al. 2002). Cellular UDP-GlcNAc needs vary in response to cell cycle stage and environmental conditions: chitin rapidly accumulates in G1 during budding (Spellman et al. 1998), in response to alpha factor (Schekman and Brawley 1979), and after cell wall disruption (Lagorce et al. 2002). Under nutrient starvation conditions, glucose may also be shunted away from this pathway. As a negative regulator of the HBP, tight regulation of Isr1 by multiple kinases may allow rapid titration of HBP activity in response to various cellular signals.

Since Pho85 and Cdk1 are activated by different cell cycle and environmental signals via their association with different cyclins, their redundant targeting of Isr1 may allow the integration of multiple environmental inputs to regulate the HBP in response to different stimuli. Precedent exists for such redundancy among CDK-related kinases. For example, the CDK-like kinase Ime2 is expressed in meiosis and is required for Sic1 destruction and the G1-S transition during meiosis (Dirick et al. 1998). In this case, Ime2 replaces the role that Cdc28 and Pho85 would play in Sic1 destruction, but only in the case of meiosis. This allows early meiotic gene expression to be coupled with DNA replication. Overlap in kinase functionality, particularly CDK-like kinases, may allow a substrate to be targeted for the same purpose at different times, providing the cell with a mechanism to dynamically control a particular cellular pathway. In the case of Isr1, Pho85 and Cdc28 may target Isr1 at different points in the cell cycle or in response to cellular stressors like glucose starvation or agents that induce a mitotic checkpoint arrest, altering metabolism as the cellular needs for UDP-GlcNAc shift.

Materials and Methods

All experiments were performed as in Alme et al. 2020.

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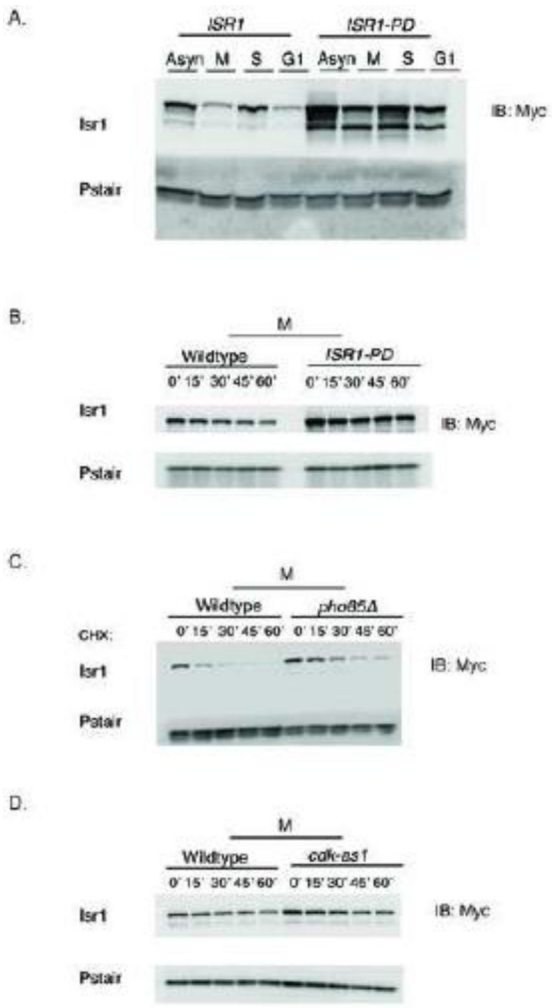

Fig 1. Cdk1 targets Isr1 in mitosis

(A) Isr1 is cell cycle regulated. Western blot showing levels of Isr1-13xmyc and Isr1-PD-13xmyc in cells growing asynchronously or arrested in G1, S, or M phase with alpha factor, Hydroxyurea (HU) or nocodazole, respectively. PSTAIR is a monoclonal antibody that recognizes the PSTAIR sequence in Cdc28 and is shown as a loading control. (B) Isr1-PD is stable during mitosis. Cycloheximide-chase assay of Isr1-13xmyc or Isr1-PD-13xmyc after release from nocodazole. Cells were treated with 15 μg/ml nocodazole 2 hours and cycloheximide was added upon release for the indicated number of minutes. Levels of Isr1-13xMyc and PSTAIR (loading control) are shown. (C) Pho85 partially stabilizes Isr1 during mitosis. Experiment was performed as in B in wildtype or *pho85*Δ cells. (D). Experiment was performed as in B in wildtype or cells expressing *cdk-as1* and treated with 10μM 1-NM-PP1 for 15 minutes before cycloheximide addition.

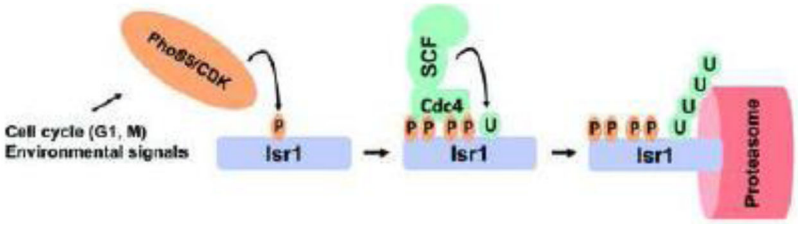

Fig 2. Cyclin Dependent Kinases target Isr1 for proteasomal degradation

Pho85 and CDK phosphorylate Isr1 in response to cell cycle or environmental signals. SCF-Cdc4 recognizes these phosphodegrons and ubiquitinates Isr1, targeting it to the proteasome.