11-20-2009

Regulation of the Alr1 transporter by Mg supply

Phaik Har Lim
University of Missouri-St. Louis, phlim84@yahoo.com

Regulation of the Alr1 transporter by Mg supply

by

Phaik Har Lim

B.Sc., Biotechnology, State University of New York, Buffalo, 2005

A THESIS

Submitted to the Graduate School at

The University of Missouri-St. Louis

In Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

in

Biochemistry and Biotechnology

December, 2009

Advisory Committee:
Colin MacDiarmid, Ph.D
Chairperson

Wendy Olivas, Ph.D
Lisa Schechter, Ph.D

ABSTRACT

Magnesium (Mg), the most abundant intracellular divalent cation, is essential for life. It has been estimated that Mg is required for the function of at least 16% of all characterized metalloenzymes. Mg ions play crucial roles in processes of cellular energy metabolism, nucleic acid and protein synthesis, nucleotide excision repair, membrane stability, cell cycle control, and hormonal regulation. While the biological importance of magnesium is clear, magnesium homeostasis is still relatively poorly understood. In the yeast *Saccharomyces cerevisiae*, Mg uptake across the plasma membrane primarily occurs via the magnesium transporter Alr1. The aim of this work was to better understand how the expression and activity of this key Mg transporter is regulated by Mg supply in yeast. Preliminary data from our laboratory and other workers had demonstrated that steady-state Alr1-HA accumulation was responsive to Mg supply. In addition, the *pep4*, *npil*, and *doa4* mutations substantially increased the accumulation of the Alr1-HA protein in replete conditions. This differential accumulation was shown to depend on the co-expression of an epitope-tagged Alr1 protein (Alr1-HA) with the unmodified Alr1 and Alr2 proteins. Based on these observations, it was suspected that the Alr1 protein was regulated post-translationally, that its regulation was dependent on ubiquitination, and that the C-terminal cytosolic domain of Alr1 was important for regulation.

To test this model, I investigated the effect of modifying the Alr1 C-terminal and N-terminal ends on the accumulation of the Alr1 protein, and used a novel antibody to compare their accumulation to the unmodified protein. Surprisingly, I observed a much less pronounced change in the accumulation of the N-terminally epitope-tagged Alr1 protein and the native (untagged) protein in response to Mg supply, and found that the accumulation of the N-terminally tagged Alr1 protein was much less dependent on co-expression with the unmodified Alr1 and Alr2 proteins than was the C-terminally tagged version. In addition, the accumulation of both untagged and N-terminally tagged Alr1 proteins was much less affected by the *doa4* and *pep4* mutations. These data suggested that the modification of the C-terminal domain of Alr1 was responsible for the majority of the differential accumulation of Alr1 in response to Mg supply. Comparison of native Alr1 accumulation with Ni^{2+} uptake assays that measure Alr system activity suggested

that both Mg deficiency and the *mnr2* mutation increased activity, but that these effects were not well correlated with Alr1 protein accumulation. For this reason, I investigated other potential mechanisms for the regulation of Alr1 activity. The Alr1 protein was suspected to be modified by phosphorylation under Mg-replete conditions. Western blot analysis showed that changes in the gel mobility of the Alr1 protein with Mg supply correlated with changes in the activity of the Alr systems, indicating that the post-translational modification of the Alr1 protein was sensitive to Mg availability, and may be involved in the regulation of Alr1 activity. As a preliminary investigation into the role of Alr1 phosphorylation, I constructed two deletions of the C-terminal domain (Δ828 and Δ806). These mutations removed two conserved serine residues that had previously been reported to be potentially phosphorylated (S847 and S850). Deletion of almost the entire C-terminal domain (Δ806) had a small effect on growth in medium with a reduced Mg concentration, and was associated with a substantial decrease in the accumulation of the protein. However, the smaller deletion did not have any effect on Alr1 function. Using fluorescence microscopy with YFP-tagged versions of the proteins, I investigated the effect of the deletions on Alr1 location. The deleted proteins (Δ828 and Δ806) accumulated slightly more in the vacuole under Mg deficient conditions, which was consistent with a minor effect of the C-terminal deletions on their stability. However, significant signal was still observed at the plasma membrane in both replete and deficient cells. Thus, these deletions provide a means to investigate the role of the C-terminal domain and the conserved serine residues in the regulation of Alr1 activity. I propose a strategy for this analysis, and describe the possible results of this work.

DEDICATION

I dedicate this dissertation to my loved ones:

- To my parents, Seng Hock Lim and Su Kim Heng.
- To my siblings, Phaik Chen, Poh Tin and Hong Woon.
- To my fiancé, Wan Rock.

ACKNOWLEDGEMENTS

This project would not have been possible without the support of numerous individuals and organizations.

I would like to express my gratitude and appreciation to my advisor, Dr. Colin MacDiarmid, for his encouragement and advice throughout my research and writing.

Special thanks to my past lab partners, Nilambari Pisat, Frank Donovan and Lauren Stein, for their help and support, and for the wonderful memories during my years in the lab.

Many thanks to the members of my thesis committee, Dr. Wendy Olivas and Dr. Lisa Schechter, for their generous gift of time, expertise, guidance and support. In particular, I wish to acknowledge Dr. Wendy Olivas for her help in submitting last minute paperwork while I was living in Texas.

Thanks are due to the many individuals who provided help and support during this research. Special thanks to Frank Donovan and Lauren Stein for making the YFP-Alr1 constructs. Thanks also to David Salt for performing the ICP-MS analysis, and Niddhi Gadhia for performing nickel uptake experiments to measure Alr system activity, and allowing me to include this data in my thesis. Thanks also to the faculty and staff of the Department of Biology for their help and guidance, in particular Maryann Hempen and Kathy Burney-Miller.

I would like to thank my friends for being there for me over the past several years in St Louis. Special thanks to Bryan Ames and Cecile Ames who provided me with support and accommodation in St Louis, and to Alex Yap and his wife, who provided help when I really needed it. Thanks also to my host family, Earl Wetter and Rinnea Wetter, for their friendship, encouragement and support.

Thanks to my family for having been so supportive and understanding during my years in the USA, and my fiancé Wan Rock for his unwavering support and love.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 1: Introduction

1.1 Importance of magnesium homeostasis

Magnesium (Mg) is the 8th most abundant element on earth and highly soluble in water. Consequently, Mg ions are relatively bioavailable (Maguire & Cowan, 2002). Perhaps because of their abundance, Mg ions are widely utilized in biology (Maguire & Cowan, 2002). However, Mg ions are not always present at concentrations ideal for life in all environments. Since Mg is essential for many biological processes, an imbalance or disregulation of magnesium in cells or whole organisms can have severe physiological consequences. In humans for example, evidence suggests that disregulation of Mg increases susceptibility to cardiovascular heart disease and may accelerate aging (Niegowski & Eshaghi, 2007; Payandeh et al, 2008). For this reason, cells and organisms possess homeostatic mechanisms to regulate the uptake and utilization of Mg. Understanding these mechanisms of Mg homeostasis could provide new insights into the biological role of Mg in metabolism and growth. The aim of this work was to better understand one aspect of Mg homeostasis in the yeast *Saccharomyces cerevisiae*, how the expression and activity of a key Mg transporter (Alr1) is regulated by Mg supply.

In **Chapter 1** of this thesis, I outline the current state of knowledge about Mg homeostasis. Because of the similarities between the physiology of yeast and plant systems, and the relatively poor understanding of Mg homeostasis in plants, this review will primarily focus on what is known about Mg homeostasis in fungal and plant systems.

In **Chapter 2**, I will detail the reagents and methods used during the performance of this work.

In **Chapter 3**, I will describe the major findings of my research, focusing on the regulation of the abundance, modification, and activity of the yeast Alr1 Mg transporter by Mg supply.

In **Chapter 4**, I will discuss the overall model of Mg homeostasis in yeast supported by my findings, and detail some future strategies to resolve uncertainties in this model and further broaden our understanding of Mg homeostasis in eukaryotes.

1.2 Biochemical properties of magnesium

Mg is the most abundant intracellular divalent cation, and essential for living cells (Laires et al, 2004; Maguire, 2006). The total cellular concentration of Mg in mammalian cells is between 14 and 20 mM (Romani & Scarpa, 1992). Magnesium is a key cofactor for a wide range of enzymes including RNA polymerases and ATPases (Laires et al, 2004). It has been estimated that Mg is required for the function of at least 16% of all characterized metalloenzymes (Waldron et al, 2009), more than twice the number that utilize zinc, the next most common metal co-factor. Mg plays crucial roles in cellular energy metabolism, DNA and chromatin structure stability, nucleic acid synthesis, nucleotide excision repair, membrane stability, cell cycle control and apoptosis, and hormonal regulation (Hartwig, 2001).

The role of Mg in biology is a consequence of not just its relative abundance, but also its specific chemical and biochemical properties. Among the biologically relevant divalent cations, Mg has the smallest naked ionic radius, but the largest hydrated radius (Kehres & Maguire, 2002; Maguire & Cowan, 2002). This difference arises because the water shell around the hydrated ion is 400x larger than the ion itself (Kehres & Maguire, 2002; Maguire & Cowan, 2002). In aqueous solutions, Mg is a hexacoordinate cation with octahedral geometry (Sreedhara & Cowan, 2002). Although Mg ions can interact with the central nitrogen atoms of a porphyrin ring [*e.g.*, in chlorophyll (Kehres & Maguire, 2002)], it prefers to associate with oxygen ligands. The distance between the magnesium ion and oxygen atom in these complexes is generally small, varying between 2.05 to 2.25 Å (Kehres & Maguire, 2002; Maguire & Cowan, 2002). For Ca^{2+}, this variation is larger (from 2.2 to 2.7 Å) (Maguire & Cowan, 2002). Significantly, Mg has less flexibility in bond angle than cations like Ca^{2+}. The rigid structure of Mg complexes is important for the correct function of magnesium-dependent enzymes (Cowan, 2002). For example, in most cells 90% of total ATP is bound to magnesium, which facilitates its biological activity. Mg binds to ATP in the catalytic pocket of ATP-dependent enzymes (Maguire & Cowan, 2002), but also coordinates one or two water molecules that play a role in phosphate ester hydrolysis (Cowan, 2002; Maguire & Cowan, 2002). For non-ATP-dependent enzymes, Mg often functions to hold a water molecule in a specific position within the active site, which facilitates the direct participation of the water

molecule in catalysis (Cowan, 2002; Maguire & Cowan, 2002).

In nucleic acid biochemistry, DNA and RNA polymerases require Mg ions to mediate nucleotidyl and phosphoryl transfer reactions (Cowan, 2002). Mg also interacts directly with the nucleic acid backbone to stabilize base pairing, and is essential for the function of catalytic RNA (Sreedhara & Cowan, 2002). Mg can thus be thought of as a "primordial" enzyme cofactor and structural element that likely played an essential role in the earliest biological processes.

1.3 Cellular distribution of magnesium

In mammalian cells, Mg is thought to be distributed evenly among the mitochondria, the endo(sarco)plasmic reticulum and the nucleus (Romani & Maguire, 2002). The majority of intracellular magnesium is bound to negatively charged ligands such as polyphosphate, acidic protein residues, nucleic acids (RNA and DNA) and nucleotides, in particular ATP (Bui et al, 1999). Only a small fraction of cellular Mg content is present as the free ionized form (Bui et al, 1999). In mammalian cells, the largest single pool of free ionized Mg is in the cytosol, where it is maintained in the range of 0.4-1 mM (Bui et al, 1999; Romani & Maguire, 2002), while some bacteria maintain a resting free ionized Mg concentration of 0.9 mM (Froschauer et al, 2004). In yeast and plant cells, a large amount of Mg is stored within large lytic vacuoles, the equivalent of the mammalian endosome (Simm et al, 2007). This vacuolar pool can be utilized to support growth in deficient conditions (Pisat et al, 2009), and may thus buffer the cytosol against changes in external Mg availability.

1.4 Magnesium and plant growth

In plants, Mg is an essential macronutrient required for normal growth and development. As in other eukaryotic cells, Mg plays a key role in the function of numerous enzymes. As consequence of its position as the central atom in the chlorophyll molecule, Mg has an important role in photosynthesis. The inclusion of Mg in chlorophyll enables the efficient capture and utilization of a broader range of light wavelengths. Mg also plays a critical role in biochemical pathways of chlorophyll biosynthesis (Shaul, 2002).

Global crop production will need to increase by 50% by 2050 to keep pace with human population growth (Flowers, 2004; Rengasamy, 2006), and Mg deficiency is one factor limiting crop productivity (Shaul, 2002). Mg deficiency is mainly associated with accelerated leaching of Mg from acidic soils (pH < 5.0), which make up approximately 40% of arable land. In general, the Mg requirement for plant growth is approximately 0.15% of dry weight. When Mg is in short supply, growth is reduced and a range of deficiency symptoms can appear. Leaf yellowing (chlorosis) of fully expanded leaves is the primary symptom of deficiency (Shaul, 2002). Light can intensify Mg-deficiency symptoms, leading to interveinal chlorosis and reddish spots on the leaves. More severe Mg deficiency and light intensity may result in necrosis (Marschner & Cakmak, 1989). Many groups have reported that Mg deficiency is associated with a decreased photosynthetic rate (Fischer & Bremer, 1993; Hermans et al, 2004; Laing et al, 2000; Sun & Payn, 1999). Deficiencies of Mg result in higher accumulation of carbohydrate (sugars and starch) in source leaves (Marschner & Cakmak, 1989), possibly as a consequence of impaired carbohydrate export from Mg-deficient leaves to sink sites. Early sugar accumulation in the Mg-deficient leaves may lead to the downregulation of the *cab2* gene that encoding Chlorophyll a/b binding protein, which in turn may trigger the decrease in content of Chl b and Chl a (Hermans et al, 2004).

In acidic soils, higher concentrations of positively charged cations such as Al^{3+}, H^+ and NH_4^+ may also inhibit Mg uptake (Mengel K, 2001; Shaul, 2002). These cations may compete with Mg for binding to negatively charged groups within the root apoplasm (Marschner, 1995). As a result of increased Al^{3+} solubility, Al toxicity is commonly encountered in crops grown in acidic soils (pH < 5.0) (Delhaize & Ryan, 1995). Al^{3+} toxicity impairs root growth, thus reducing yields (Poschenrieder et al, 2008) and is associated with Mg deficiency (Grimme, 1983). Mg additions were reported to alleviate Al^{3+} toxicity and Al-induced Mg deficiency in a wide range of plant species (Keltjens & Tan, 1993; Matsumoto, 2000; Silva et al, 2001a; Silva et al, 2001b). For example, application of Mg to soils was reported to reduce Al^{3+} toxicity in several plant species, including wheat (Ryan et al, 1994) and soybean (Silva et al, 2001a; Silva et al, 2001b). These observations have led to the suggestion that the symptoms of Al toxicity are in part caused by Mg deficiency. Some workers have proposed that Al^{3+} toxicity occurs as a

consequence of the inhibition of Mg uptake systems by the Al^{3+} ion, which is similar in size to the hydrated Mg ion. In support of this model, the overexpression of genes encoding the Alr1 and Alr2 Mg channels conferred increased Al^{3+} tolerance in yeast cells, possibly by increasing the cells capacity for Mg uptake in the presence of a normally inhibitory concentration of Al^{3+} (MacDiarmid & Gardner, 1996; MacDiarmid & Gardner, 1998a). Although this work was performed in yeast, a recent study reported that the overexpression of an Arabidopsis homology of the Alr proteins (AtMgt1) in Tobacco conferred significant Al^{3+} tolerance (Deng et al, 2006), indicating that at least in some plant species, Al^{3+} toxicity and Mg supply may be connected.

Although essential, excess Mg can also be toxic to plants due to the inhibition of photosynthesis and plant growth. The effect of excess Mg is exacerbated by water stress [reviewed in (Shaul, 2002)]. High Mg content together with dehydration in plants was reported to inhibit photosynthesis (Rao et al, 1987). Hence, Mg homeostasis is essential for plant health.

1.5 Magnesium transporters

The highly charged nature of Mg ions means that integrated transporter proteins are required to facilitate its passage across hydrophobic membranes. These proteins determine the distribution of Mg between the cell, environment, and membrane-bound cellular compartments. For this reason, studies of Mg homeostasis have initially focused on the identification and characterization of these proteins. The large size of hydrated Mg ions, and the strong bond between Mg ions and water molecules impose interesting mechanistic problems for proteins that transport Mg through biological membranes. In addition, the strong electrical driving force for Mg uptake by cells adds to the problem of homeostasis, since Mg influx via specific transporters has to be tightly controlled, and efflux mechanisms must exist to remove Mg that leaks in via non-specific pathways. Some of the major families of transporters that have been found in bacteria, fungi and plant systems are described below.

1.5.1 Mg transporters in prokaryotes

1.5.1.1 CorA

Named for the cobalt-resistant mutants in which it was first identified, CorA and its homologs are widespread in eubacteria and archaea (Hmiel et al, 1986; Kehres et al, 1998). CorA, an integral protein of the bacterial plasma membrane, is responsible for magnesium influx, and can also mediate efflux under specific conditions (Smith et al, 1993; Smith & Maguire, 1998). Besides its role as Mg transporter, it is capable of transporting additional divalent cations, including Co^{2+} and Ni^{2+} (Hmiel et al, 1986). Among these three divalent cations, CorA has highest affinity for Mg (K_m of 15-20 μM), and its ability to mediate transport of other substrates is thought to have little physiological significance. However, its relatively broad specificity means that CorA confers sensitivity to high concentrations of Co^{2+} and Ni^{2+} in bacteria such as *S. typhimurium* (Kehres & Maguire, 2002).

The CorA protein has a hydrophilic N terminal region located in the cytosol, and a relatively hydrophobic C-terminal region with two TM domains in the periplasmic membrane (Payandeh & Pai, 2006a). The overall sequence homology between CorA family members in prokaryotes and eukaryotes is low (Kehres & Maguire, 2002; Maguire, 2006), and is highest in the C-terminal region (Niegowski & Eshaghi, 2007). The CorA gene family is defined by the presence of two transmembrane domains (TM-1, TM-2) near to the C terminus, and a glycine-methionine-asparagine (GMN) motif at the C-terminal end of TM-1. This highly conserved GMN motif is essential for Mg transport (Knoop et al, 2005). Recently, several groups determined the atomic structure of a CorA protein from *Thermatoga maritima* (Eshaghi et al, 2006; Lunin et al, 2006; Payandeh & Pai, 2006a). These structures indicated that five CorA monomers form a pentamer consisting of two major domains, a large N-terminal "funnel" with a highly polar interior, and a membrane-spanning pore, with its inner face formed by the N-terminal transmembrane domain of each subunit. These structures also revealed the presence of several apparent Mg-binding domains within the funnel, which may act to regulate the conformation and permeability of the pore according to the cytosolic Mg concentration. Genetic studies indicated that residues involved in binding Mg at these sites were

critically important for the regulation of channel activity (Payandeh et al, 2008).

1.5.1.2 CorB, CorC and CorD

S. typhimurium cells loaded with radioactive Mg displayed Mg efflux activity. This process was dependent on a relatively high concentration of external Mg (above 10 mM). The physiological significance of this activity is not yet clear, although it may act to prevent overaccumulation of Mg when present in excess. The efflux activity was dependent on CorA, as it was lost in *corA* mutant strains (which retain Mg uptake capacity due to the Mgt systems). This effect could simply reflect the ability of CorA to mediate excess Mg uptake under these conditions, which was then removed from the cell via others systems. Consistent with this interpretation, efflux was shown to be dependent on at least three unlinked loci, the *CorB*, *CorC* and *CorD* genes (like CorA, these genes were originally isolated on the basis of increased cobalt resistance). Mutants lacking any single gene were still capable of Mg efflux, but only at much higher external Mg concentrations (Gibson et al, 1991). Very little is known about the function of these genes in bacteria, although the products of the CorB-D loci have been identified, revealing homology between the CorB and CorC proteins. Homologs of CorB/C (the ancient conserved domain protein, or ACDP family) have been identified in most eukaryotic genomes (Wang et al, 2004), and a vertebrate homolog was recently implicated in Mg homeostasis.

1.5.1.3 Mgt family of P-type ATPase proteins

Unlike CorA, the MgtA/B class of Mg transporters are primarily found in the *Eubacteria* (Maguire, 2006). In *S. typhimurium*, the *mgtA* and *mgtCB* loci encode Mg-transporting P-type ATPase enzymes (Hmiel et al, 1986; Smith & Maguire, 1998; Snavely et al, 1989; Snavely et al, 1991a; Snavely et al, 1991b; Tao et al, 1995). The *mgtCB* locus is a two-gene operon including *mgtC*, which encodes a membrane protein of unknown function. The absence of MgtC does not elicit any significant effect on MgtB transport capacity (Snavely et al, 1991a; Snavely et al, 1991b; Tao et al, 1995), and this protein is instead thought to contribute to pathogenicity. P-type ATPase proteins mediate high-affinity energy-dependent transport of their substrates (Carafoli & Brini, 2000; Scarborough, 1999). While some members of this family mediate metal efflux, MgtA and

MgtB both mediate Mg influx, and are unable to mediate efflux (Hmiel et al, 1989; Snavely et al, 1989). This property of the transporters has lead to suggestions that they participate in scavenging Mg from environments in which it is present at very low concentrations, for example when bacteria are engulfed by macrophages. The observation that the *mgt* genes are members of the PhoP/Q regulon, which includes genes required for the pathogenicity of *S. typhimurium,* indirectly supports this model (Groisman, 2001).

1.5.1.4 MgtE

The first member of the MgtE class of Mg transporters was cloned from the Gram-positive alkaliphile *B. firmus* OF4 (Smith et al, 1995) and the Gram-negative *P. stuartii* (Townsend et al, 1995). The expression of MgtE in a *corA mgtA mgtB* strain restored growth and allowed Mg uptake. MgtE homologs are widespread among *Eubacteria* (Maguire, 2006). Interestingly, many if not most bacterial species possess both a CorA and a MgtE homolog, indicating that these proteins may play different roles in Mg homeostasis.

1.5.2 Mg transport in eukaryotes

1.5.2.1 Fungal transporters

Several yeast CorA homologs have been identified in the plasma membrane (Alr1 and Alr2), mitochondrial inner membrane (Mrs2 and Lpe10) and vacuole membrane (Mnr2). The first of these proteins identified were Alr1 and Alr2, named for their ability to confer resistance to a high concentration of aluminum ion (Al^{3+}) when overexpressed. This phenotype probably arises as a consequence of the ability of Al^{3+} ions to inhibit Mg uptake via the Alr systems. In the yeast *Saccharomyces cerevisiae*, Mg uptake across the plasma membrane primarily occurs via the Alr1 protein (Graschopf et al, 2001; MacDiarmid & Gardner, 1998a), although Alr2 also contributes in some genetic backgrounds (Pisat et al, 2009). Membership of the Alr proteins in the CorA family is supported by the presence of two transmembrane domains near the C terminus, and the GMN motif located at the end of the first C-terminal TM domain. Functional homology of Alr1 and CorA is supported by the observation that expression of bacterial CorA could restore the growth defect associated with the *alr1* mutation in yeast (Graschopf et al,

2001). Overexpression of Alr2 can compensate for the absence of Alr1, again indicating that these proteins overlap in function (MacDiarmid & Gardner, 1998a).

1.5.2.1.1 Mrs2 and Lpe10

The *MRS2* gene was first identified as a suppressor of a mitochondrial Group II intron splicing defect (Wiesenberger et al, 1992). Later studies indicated that Mrs2 has a primary role in mitochondrial Mg homeostasis (Bui et al, 1999; Wiesenberger et al, 1992), and is required for activity of the major Mg influx system in the mitochondrial inner membrane of yeast (Kolisek et al, 2003; Schindl et al, 2007). The steady-state concentration of mitochondrial Mg is dependent on the presence of Mrs2 (Kolisek et al, 2003). Single channel patch clamping experiments showed that Mrs2 forms a high conductance Mg-selective channel in the inner mitochondrial membrane. Consistent with Mrs2 homology to CorA, Mg influx is inhibited by Co(III)hexaamine (Schindl et al, 2007). A close homologue of Mrs2, Lpe10, is also located on the mitochondrial inner membrane, and is equally important for mitochondrial Mg homeostasis (Bui et al, 1999; Gregan et al, 2001). Mrs2 or Lpe10 overexpression leads to a moderate increase in the mitochondrial Mg concentration (Bui et al, 1999; Gregan et al, 2001). In contrast, disruption of either gene causes a growth defect in cells supplied with nonfermentable carbon sources, and a significant reduction of the intramitochondrial Mg concentration (Bui et al, 1999; Gregan et al, 2001). These defects could be partially restored by the overexpression of CorA, indicating that Mrs2 and Lpe10 are functional homologs of CorA (Bui et al, 1999; Gregan et al, 2001).

1.5.2.1.2 Mnr2

Mnr2 is the fifth CorA homolog in *S. cerevisiae* (Pisat, 2009). Like other CorA members, Mnr2 has two transmembrane domains and a GMN motif; however, it has a tryptophan residue (W) preceding the GMN motif (Knoop et al, 2005; MacDiarmid & Gardner, 1998a). The possession of this conserved residue suggests that the Mnr2-like group performs a different function to the Alr1-like group of proteins (Pisat, 2009). Mnr2 is located in the vacuole membrane, suggesting that it is required for the release of vacuolar Mg under deficient conditions. Consistent with this model, a Mg-replete *mnr2* strain accumulated more Mg than wild-type, but this increased content did not promote

growth in Mg-deficient conditions. Overexpression of Mnr2 redirected the protein to the plasma membrane, where it could suppress the growth defect of an *alr1 alr2* mutant. Overall, the data suggests that Mnr2 is capable of mediating Mg transport. Increased accumulation of divalent cations such as Mn^{2+} and Co^{2+} was observed in *mnr2* mutant cells, indicating that the *mnr2* mutation triggered an increase in the expression or activity of Alr1 and Alr2. Consistent with this model, the mutant was more sensitive to these cations. This observation suggests that the activity of the Alr systems may be coupled to cytosolic Mg concentration.

1.5.2.2 Mg transporters of higher plants

1.5.2.2.1 AtMRS/AtMGT family

The AtMRS/AtMGT family, a eukaryotic subset of the CorA superfamily, comprises ten members in *Arabidopsis thaliana* (Li et al, 2001b; Schock et al, 2000), and was independently described by two laboratories. AtMRS2-1 and 2-2 were identified by their similarity to the *Saccharomyces cerevisiae MRS2* gene, while AtMGT1 and AtMGT10 were identified via their ability to rescue the growth defect of a yeast *alr1 alr2* mutant (Li et al, 2001b; Schock et al, 2000). Li and colleagues designated AtMRS2-1 and AtMRS2-2 as AtMGT2 and AtMGT9 respectively (Li et al, 2001b). AtMRS2-1 was also shown to complement an *mrs2* yeast mutant (Schock et al, 2000), while expression of AtMGT1 in a *corA mgtA mgtB* triple mutant of *S. typhimurium* complemented its growth defect in media containing 10 μM Mg (Li et al, 2001b). AtMGT1 preferentially transported Mg, but was also capable of mediating Ni^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, and Cu^{2+} transport, and was inhibited by Cobalt (III)-hexaamine. Another member of the AtMGT family, AtMGT7a, appears to be a low-affinity Mg transporter (Mao et al, 2008). AtMGT7a is a product of the alternative splicing of the AtMGT7 gene. Expression of AtMGT7a could suppress the growth defect of a *corA mgtA mgtB* mutant, but the strain required a higher Mg concentration (500 μM Mg) for full complementation. Together, these data suggested that several members of the AtMGT gene family are functional homologs of CorA (Li et al, 2001a).

Based on their sub-cellular location, members of the AtMGT family play diverse roles in magnesium homeostasis in plants. AtMGT1 was localized to the plasma

membrane of cells (Li et al, 2001a), where it presumably plays a role in obtaining Mg from the environment. In contrast, AtMGT10 (AtMRS2-11) was located in the chloroplast envelope membrane system (Drummond et al, 2006), and showed diurnal regulation of gene expression, although its potential role in maintaining chloroplast Mg content has not yet been directly established. Another protein, AtMGT5, was found to be located in the mitochondrial membrane (Li et al, 2008), and to be essential for pollen viability. Thus far, a member of this family located in the tonoplast has yet to be identified.

1.5.2.2.2 Biotechnological applications of Mg transporters

Studies of these Mg transporters have revealed potential biotechnological applications. For example, the overexpression of AtMGT1 in *N. benthamiana* increased Al^{3+} tolerance (Deng et al, 2006). Roots of AtMGT1-overexpressing transgenic plants showed improved growth in Al^{3+} concentrations where root growth was completely inhibited in the wild type. In line with this observation, the root tips of transgenic plants exhibit significantly lower deposition of callose (an indicator of Al^{3+}-induced stress) under the same conditions. The result is consistent with previous data showing that the overexpression of Alr1 or Alr2 could confer Al tolerance in yeast cells by increasing the capacity for Mg uptake (MacDiarmid & Gardner, 1996; MacDiarmid & Gardner, 1998a). Al toxicity is commonly encountered in crops grown in acidic soils (pH<5.0) as a result of increased Al^{3+} solubility (Delhaize & Ryan, 1995). The high Al^{3+} concentration in acidic soils impairs root growth, thus reducing yields (Poschenrieder et al, 2008). The observation that manipulation of Mg supply or transporter activity could suppress Al^{3+} toxicity is an important advance in the development of agricultural strategies to improve yields on acidic soils.

1.5.2.3 Mg transport in vertebrates

1.5.2.3.1 TRPM6 and TRPM7

In animals, correct magnesium status is primarily maintained by regulating the rates of both intestinal absorption and renal excretion (Schmitz et al, 2007). The disturbance of magnesium homeostasis has a variety of detrimental effects on animals.

For example, many diseases such as hypertension, cardiac arrhythmia, diabetes and chronic alcoholism are associated with diminished Mg levels (Schmitz et al, 2007; Touyz, 2008). Understanding how these syndromes occur requires knowledge of the systems which maintain Mg flux in and out of vertebrate cells. Some of the most important of these systems are discussed below.

TRPM7 and TRPM6, the first vertebrate Mg transporters identified, were originally cloned in a screen for novel kinases (Ryazanov, 2002). In subsequent studies, both proteins were found to play roles in Mg homeostasis (Hoenderop & Bindels, 2005; Konrad et al, 2004; Montell, 2003; Schmitz et al, 2004; Wolf, 2004). The TRPM family of ion channels was named for the melastatin 1 (TRPM1) protein, a potential tumor suppressor in malignant melanoma cells (Xu et al, 2001). Two TRPM members, TRPM6 (CHAK2) and TRPM7 (CHAK1), share the unique feature of a C-terminal α-kinase catalytic domain fused to the TRP ion channel (Schmitz et al, 2007).

TRPM7 appears to play an important role in magnesium homeostasis (Schmitz et al, 2003). Targeted gene deletions of TRPM7 in DT-40 avian lymphocytes resulted in Mg deficiency and growth arrest (Schmitz et al, 2003). The TRPM6 protein also has a role in epithelial magnesium reabsorption (Schlingmann et al, 2002; Walder et al, 2002). Despite their high sequence similarity, the TRPM proteins are functionally non-redundant (Nilius et al, 2007a; Nilius et al, 2007b). The hypomagnesemic phenotype of TRPM6-deficient patients is not prevented by the presence of the ubiquitously expressed TRPM7 (Schlingmann et al, 2007). In addition, heterologously expressed TRPM6 could not compensate for TRPM7 deficiency in DT40 cells (Schmitz et al, 2005). These results suggest that the proteins play non-overlapping roles in Mg homeostasis (Schlingmann et al, 2007).

While TRPM7 is ubiquitously expressed, TRPM6 is mainly expressed in the distal convoluted tubule kidney and the intestines (Chubanov et al, 2004; Tiel Groenestege et al, 2006). Under physiological conditions, TRPM7 was primarily selective for Mg and did not transport Ca^{2+} and other divalent cations (Touyz, 2008). In support of the physiological role of TRPM6 in Mg homeostasis, mutations in the TRPM6 loci are responsible for the human genetic disease primary hypomagnesaemia with secondary hypocalcemia (HSH). Patients with this disorder display a combined defect in

intestinal magnesium absorption and renal magnesium conservation (Schlingmann et al, 2002; Walder et al, 2002). Despite these findings, however, more recent reports have downplayed the role of the TRPM proteins in Mg homeostasis, citing the extremely low conductance of these channels and their relative lack of metal specificity as evidence that their primary biological role has yet to be identified.

1.5.2.3.2 Novel vertebrate Mg transporters

Recently, several studies have identified novel proteins that may play a role in Mg homeostasis in vertebrates. One such protein is MagT1 (Goytain & Quamme, 2005c). Expression of this protein is regulated by Mg supply (Goytain & Quamme, 2005c; Zhou & Clapham, 2009), and the gene was originally isolated as one of a set upregulated by Mg deficiency in cultured kidney cells. MagT1 is a relatively hydrophobic protein with homology to the yeast Ost3 and Ost6 proteins, thought to be components of a complex required for N-glycosylation in the ER (Yan & Lennarz, 2005). MagT1 expression in yeast complemented the growth defect of a *Saccharomyces cerevisiae alr1* mutant (Zhou & Clapham, 2009), suggesting that this protein is a genuine Mg transporter. MagT1 knockdown significantly reduced both free and total intracellular Mg content in mammalian HEK 293T cells, suggesting that it plays an important role in Mg uptake. Consistent with this finding, MagT1 appears to be widely expressed in human and mouse tissues (Goytain & Quamme, 2005c). Several additional Mg-regulated genes encoding apparent membrane proteins were identified by the same method that used to find MagT1 (Goytain & Quamme, 2005a; Goytain & Quamme, 2005b; Goytain & Quamme, 2005c). The SLC41A1, SLC41A2, and ACDP2 proteins formed Mg channels when expressed in *Xenopus* oocytes. Although characterization of these proteins is still in its early stages, several reports suggest that the SLC proteins in particular play important roles in Mg homeostasis (Goytain & Quamme, 2005b; Kolisek et al, 2008).

Despite progress in the identification of Mg channels, a protein that mediates active transport of Mg in vertebrates has yet to be definitively identified. However, one report suggests that the SLC41A1 protein is capable of mediating Mg efflux from cultured mammalian cells under some conditions, although the mechanism of this process is unclear (Kolisek et al, 2008). Some reports have implicated members of the cation

exchanger (CAX) superfamily in Mg transport. This superfamily of proteins includes transporters specific for various divalent and monovalent cations (Grinstein & Wieczorek, 1994; Maser et al, 2001). Two human Ca^{2+}/Na^{+} exchangers, Ncx1 and Ncx3, were reported to mediate Mg efflux when overexpressed (Tashiro et al, 2000). However, this work has not been repeated. Another CAX family protein, XntA from *Paramecium tetraurelia*, forms a Mg-selective channel in the plasma membrane, but does not mediate active transport (Haynes et al, 2002). Nevertheless, the diversity of metal ions transported by this large group of proteins suggests that it may well include a member capable of active Mg transport.

1.6 Regulation of magnesium transporter activity

The disturbance of Mg homeostasis causes a variety of severe detrimental effects on cells, including human cells. For example, Mg deficiency in human is associated with hypertension, cardiac arrhythmia, diabetes and chronic alcoholism. However, Mg may also be toxic when present in excess. Perhaps as a consequence of this requirement, the expression, stability or activity of several Mg transporters has been shown to be regulated by Mg supply. The following section describes how this regulation was shown to be achieved.

1.6.1 Transcriptional regulation of magnesium transporters

1.6.1.1 MgtA and MgtB

The expression of both the MgtA and MgtB genes is regulated according to external Mg availability. Both genes are induced at least 1000-fold when cells are grown in medium with a low Mg concentration (Garcia Vescovi et al, 1996; Tao et al, 1998; Tao et al, 1995). MgtA/B regulation is dependent on the two-component signal transduction system PhoPQ (Groisman et al, 1989; Miller, 1991). In the presence of high extracellular Mg concentrations, Mg binds to the PhoQ membrane sensor kinase and changes its conformation (Garcia Vescovi et al, 1996). PhoQ transduces this signal to the soluble cytosolic PhoP transcription factor, which represses the transcription of genes regulated by Mg supply. Conversely, when external Mg is low, the repression is relieved and both transporters are expressed (Tao et al, 1998; Tao et al, 1995). Interestingly, the

transcription of a number of other genes is also induced by low extracellular Mg via the PhoPQ system (Groisman, 2001; Moncrief & Maguire, 1998). Many of these genes are required for pathogenicity of *S. typhimurium*, leading to the model that Mg deficiency represents an external signal for the expression of genes required for bacterial survival inside macrophages (Groisman, 2001).

More recently, additional mechanisms for regulation of the Mgt genes have been described. The accumulation of the MgtA mRNA is regulated by a structure formed by the 5'-UTR of the MgtA transcript (Cromie et al, 2006). This structure forms in the presence of Mg, and acts to prevent the elongation of the MgtA transcript. This provides an additional control on MgtA transcript accumulation that is linked to the intracellular, rather than extracellular Mg concentration. In addition to this regulation, it was recently shown that the stability of the MgtA transcript responds to Mg supply via the same Mg-responsive domain, which targets the mRNA for degradation when Mg concentration is high (Spinelli et al, 2008). Thus, the level of MgtA mRNA is under tight control by Mg supply, highlighting the importance of this regulation.

1.6.1.2 Transcriptional regulation of *ALR1* and *ALR2*

Expression of the major yeast Mg influx system, Alr1, was reported to be regulated by Mg supply in yeast (Graschopf et al, 2001). RT-PCR analysis of the *ALR1* transcript indicated that it also accumulated to higher levels during deficiency. Currently, no report has discussed the mechanism of this apparent transcript regulation, but a more recent report also described apparent transcript regulation of the homologous *ALR2* gene, also based on RT-PCR analysis (Wachek et al, 2006).

1.6.1.3 Transcriptional regulation of vertebrate transporters

As previously described, transcription of MagT1 is highly regulated by the extracellular Mg concentration (Goytain & Quamme, 2005c; Zhou & Clapham, 2009). The transcription of MagT1 was increased about 7.5-fold in low-Mg conditions (Zhou & Clapham, 2009). In addition to this protein, several other putative Mg transporters have been identified on the basis of the regulation of their transcript accumulation by Mg supply, and subsequent electrophysiological studies in Xenopus oocytes have identified specific roles in Mg transport for some of these proteins. No analysis of the mechanism

of this regulation has yet been presented.

1.6.2 Post-translational regulation of magnesium transporters

1.6.2.1 Regulation of Alr1 stability

The Alr1-HA protein was reported to show Mg-dependent regulation of its stability (Graschopf et al, 2001). This process was dependent on factors implicated in the post-translational regulation of other regulated transporters, including the Rsp5, End4, and Pep4 proteins. Mutants lacking these proteins have defects in the ubiquitination, endocytosis and vacuolar degradation of plasma membrane proteins respectively (see below for a discussion of the role of these proteins). How Mg supply was linked to Alr1 protein stability was not described.

In addition to the above regulation, it was also reported that Alr1 shows an apparent post-translational modification that is correlated with Mg supply. In Mg-replete cells, the protein is present as a form with lower mobility in SDS-PAGE. This form could be converted to the higher mobility form by treatment with lambda phosphatase, suggesting it occurs as a consequence of phosphorylation (Wachek et al, 2006). What role (in any) this modification plays in regulation is currently unclear. In addition, it is not yet clear that Alr1 activity changes with Mg supply, as would be expected from the above reports. This deficiency is in part due to a lack of simple methods for the measurement of Alr1 activity (due to their very short half-life, no radioactive isotopes of Mg are currently commercially available).

1.6.2.2 Higher eukaryotic transporters

Many studies have been performed to examine the post-translational regulation of Mg channel activity in vertebrate systems. Post-translational regulation of transporters can take many forms, including covalent modification of the protein (*e.g.* by addition of ubiquitin, or phosphorylation), interactions with other regulatory factors, or the intrinsic regulation of activity by metals ions. Some examples of these kinds of regulation are discussed below.

1.6.2.2.1 Regulation by intrinsic Mg-binding domains

TRPM6 and TRPM7 activity is regulated by changes in cytosolic Mg or Mg-ATP [Reviewed in Touyz, 2008 #7150]. Intracellular Mg inhibits TRPM7 activity, which may help cells to regulate cytosolic Mg concentration (Monteilh-Zoller et al, 2003; Nadler et al, 2001; Schmitz et al, 2003). The inhibitory binding site for magnesium might be at the kinase domain. The TRPM proteins have intrinsic α-kinase domains, which have been shown to phosphorylate several substrates including anexin I and myosin heavy chain. The embryonic lethality of TRPM7 mutations in mice suggests that this function is essential for development (Sontia & Touyz, 2007). A mutant of TRPM7 with its kinase domain deleted showed an altered sensitivity for channel activation in response to intracellular Mg (Schmitz et al, 2003). Consistent with this observation, a missense mutation (T1482I) in the kinase domain which eliminated activity also increased Mg sensitivity of the channel (Hermosura et al, 2005). Interestingly, it was also shown that TRPM6 could increase TRPM7 channel activity by phosphorylating TRPM7 (Schmitz et al, 2005; Schmitz et al, 2004). The TRPM6/7 proteins may serve both as magnesium uptake mechanisms and as magnesium sensors, as the channel can conduct Mg while its kinase domain modulates sensitivity to intracellular Mg (Penner & Fleig, 2007; Schmitz et al, 2004).

Another well characterized vertebrate transporter is the hsMrs2 protein, which mediates Mg uptake by mitochondria. Activity of this system has been shown to be directly regulated by matrix Mg concentration (Schindl et al, 2007). hsMrs2 is a CorA homolog, and the observed Mg-dependent gating of its activity has also been observed in bacterial CorA homologs. Mutation of the Mg-binding sites observed in the cytosolic domain of the *T. maritima* CorA protein affected the conformation and activity of this protein, supporting their role as components of a Mg-sensor domain.

1.7 Regulation of membrane protein stability by ubiquitination

The observation that Alr1 stability is dependent on factors required for membrane protein ubiquitination and stability suggests that regulation of Alr1 may be achieved via similar pathways to those observed for other regulated membrane proteins. To provide background information on these processes, some key examples of these proteins and the factors which regulate them are discussed below.

1.7.1 Regulation of Gap1 amino acid permease

Gap1 is a high capacity amino acid permease in yeast that provides an excellent example of a post-translationally regulated membrane protein (Jauniaux & Grenson, 1990; Wiame et al, 1985). Since Gap1 is only required when amino acids are unavailable, the synthesis and intracellular trafficking of Gap1 is tightly controlled according to the nitrogen source (Grenson, 1992; Roberg et al, 1997; Stanbrough & Magasanik, 1995; Wiame et al, 1985). In cells supplied with a favorable nitrogen source, Gap1 activity is low. However, in cells grown on a relatively poor nitrogen source (urea or proline), synthesis of Gap1 is activated and the protein sorted to the plasma membrane (De Craene et al, 2001; Grenson, 1983). The initial synthesis of Gap1 is controlled transcriptionally, and is subject to nitrogen catabolite repression. However, a mechanism of post-translational regulation also operates to control the level of the protein at the cell surface. When a good nitrogen source (*e.g.* ammonium) becomes available to nitrogen-starved cells, Gap1 is ubiquitinated on two lysine residues at positions 9 and 16 (De Craene et al, 2001), leading to its inactivation via the endocytic pathway (Hein et al, 1995; Springael & Andre, 1998), and degradation of the permease in the vacuole (Springael & Andre, 1998). Ubiquitination of Gap1 requires the product of the *NPI1* gene, Rsp5 (Hein et al, 1995), while Gap1 internalization and degradation requires factors essential for endocytosis (End3 and End4), and vacuolar proteolysis (Pep4).

1.7.2 Factors required for post-translational regulation

1.7.2.1 Ubiquitin

Ubiquitin (Ub) is a 76-amino acid polypeptide that can be covalently attached to a target substrate by an isopeptide bond between the C-terminal Gly-76 of ubiquitin and a Lys residue in the target. Ubiquitination serves as a signal for plasma membrane proteins to undergo endocytosis and subsequent degradation in the lysosome/vacuole. In addition to its role in Gap1 regulation, ubiquitination is essential for the endocytosis of the uracil permease Fur4, the galactose permease Gal2 (Soetens et al, 2001), and the zinc transporter Zrt1 (Gitan & Eide, 2000).

1.7.2.2 Ubiquitin ligases

Ubiquitination is an ATP-dependent reaction, which occurs through the sequential actions of three classes of enzymes: E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases. E3 proteins are believed to play an important role in defining the specificity of the substrate in the ubiquitination pathway. An important E3 ligase in yeast is the Rsp5 protein, which is encoded by *NPI1*, an essential gene (Soetens et al, 2001). The *npi1* allele is a promoter mutation that reduces the expression of this gene, allowing viability but inhibiting many regulatory processes that depend on full Rsp5 activity (Hein et al, 1995). The best documented role of Rsp5 is to regulate the endocytic pathway and to target many plasma membrane proteins for degradation in the vacuole. Rsp5 has been shown to be involved in the down-regulation of transporters such as Fur4p, Gap1p, Tat2p, and Zrt1, as well as hormone receptors like Ste2p and Ste3p (Dunn & Hicke, 2001; Galan & Haguenauer-Tsapis, 1997; Gitan & Eide, 2000; Springael & Andre, 1998). Rsp5 also participates in mechanisms of membrane protein quality control in the secretory pathway (Pizzirusso & Chang, 2004).

1.7.2.3 Ubiquitin isopeptidases

The Doa4 protein is another yeast protein that is required for the post-translational regulation of some membrane proteins (Amerik et al, 2000; Swaminathan et al, 1999). Doa4 is a ubiquitin hydrolase that removes Ub intact from membrane proteins (Swaminathan et al, 1999). Doa4 is located in the prevacuolar compartment (PVC), where it participates in the formation of multi-vesicular bodies (MVB's) (Amerik et al, 2000). MVB's are compartments that contain multiple vesicles formed by budding of membranes into the lumen of the compartment. Proteins targeted for vacuolar degradation are sorted into these vesicles, which are then delivered to the lumen of the vacuole for degradation (a Pep4-dependent process). Doa4 acts to recycle Ub from membrane proteins before they are packed into MVB's. In the absence of Doa4 activity, some membrane proteins are not delivered into MVB's, and instead accumulate on the vacuole membrane (for example, the Cps1 protein) (Morvan et al, 2004). Under appropriate conditions, the accumulated protein may be detected as the Ub-conjugated form. A side effect of the *doa4* mutation is a general decrease in free Ub, which may

inhibit the addition of Ub to newly synthesized proteins (Swaminathan et al, 1999). However, it was recently argued that the effect of the *doa4* mutation on membrane protein stability is primarily due to the involvement of Doa4 in protein sorting at the PVC, because (among other reasons) the overexpression of ubiquitin cannot compensate for the absence of Doa4 activity (Nikko & Andre, 2007).

1.8 Preliminary analysis of Alr1 regulation

Before I began the work described here, other students in the MacDiarmid laboratory (Abhinav Pandey, Aandahl Achari and Nilambari Pisat) had performed several experiments to investigate some aspects of *ALR1* regulation, providing some surprising results. For example, in contrast to a previous report (Graschopf et al, 2001), *ALR1* mRNA accumulation was found to be unaffected by Mg supply (A. Achari, C. MacDiarmid, unpublished data), as was the activity of an *ALR1* promoter-*lacZ* reporter gene (Pisat, 2009). These observations indicate that the *ALR1* gene is not transcriptionally regulated by Mg supply. However, the accumulation of an epitope-tagged version of the Alr1 protein, Alr1-HA, was found to be affected by Mg supply (Pisat, 2009). Further experiments suggested that this apparent regulation was not achieved via an effect of Mg supply on the rate of Alr1 internalization from the plasma membrane to the vacuole. In contrast to a previous report (Graschopf et al, 2001), when WT cells expressing Alr1-HA were grown in a medium containing 5 μM Mg, then transferring to media containing low (5 μM) or high (10 mM) Mg with cycloheximide to prevent *de novo* protein synthesis, the steady-state level of the Alr1-HA protein was constant for up to 3 hours in both conditions (Pisat, 2009). To further examine the regulation of Alr1-HA accumulation, other workers determined the effect of mutations in factors required for the delivery of plasma membrane proteins to the vacuole (A. Pandey, C. MacDiarmid, unpublished data). Mutants defective for endocytosis (*end3* and *dim1*) still showed differential accumulation of Alr1-HA in Mg-replete vs deficient conditions, indicating that endocytosis was not required for this effect. However, mutants deficient in the ubiquitination and trafficking of membrane proteins from the plasma membrane or late Golgi to the vacuole for degradation (*npi1*, *doa4*), or defective for the maturation of vacuolar proteases required for degradation of luminal proteins (*pep4*) showed strongly

enhanced accumulation of Alr1-HA in both conditions (A. Pandey, C. MacDiarmid, unpublished data). Since both *npi1* and *doa4* are involved in regulating the ubiquitination of membrane proteins, the effect of the mutations on differential accumulation of Alr1-HA indicated that this process may be dependent on Alr1 ubiquitination. If so, these results suggested that in Mg-replete conditions, Alr1 was targeted directly from the Golgi to the vacuole for degradation, while in deficient conditions, it was targeted to and accumulated at the plasma membrane. Furthermore, the results suggested that this apparent regulatory process was dependent on the ubiquitination either of Alr1, or of another factor that was required for its degradation.

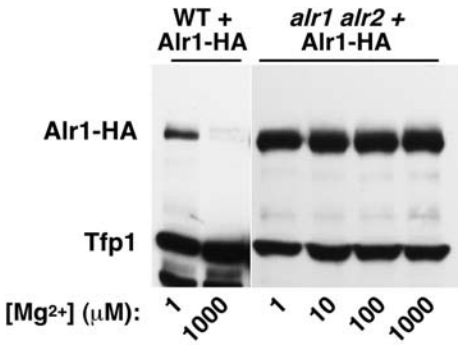

Figure 1.1. Differential accumulation of Alr1-HA is dependent on *ALR* genotype. Expression of Alr1-HA in strains of the indicated genotypes after growth in medium containing the indicated Mg concentrations. Alr1-HA and Tfp1 (a loading control) were detected by immunoblotting of total protein extracts (A. Pandey and C. MacDiarmid, unpublished data).

Subsequent work by Abhinav Pandey provided a possible clue to the location of a region important for the differential accumulation of Alr1. Our studies of Alr1 regulation have utilized a version of Alr1 with three repeats of the HA epitope tag fused to the C-terminal end of the protein. When this protein was expressed in a WT strain, which also expressed the untagged Alr1 and Alr2 proteins, differential accumulation was observed (**Figure 1.1**). However, the expression of this protein in an *alr1 alr2* mutant eliminated this difference. One possible model for this phenomenon is that the wild-type version of Alr1 or Alr2 might confer instability on the Alr1-HA protein under Mg-replete conditions. This effect could be mediated via the formation of heteromeric complexes between the HA-tagged Alr1 protein and the unmodified Alr1 and Alr2 proteins (Wachek et al, 2006). If the HA tag prevents a normal regulatory process that is required for the degradation of Alr1, then complexes made up entirely of the modified subunit would be more stable. The regulatory function might however be supplied *in trans* by the association of a normal subunit with a modified subunit within the heteromeric complex.

1.9 Objectives of this research

It is clear from studies in vertebrate systems that the expression of Mg transporters is regulated at many levels, including transcriptional. However, very little is known about how cells sense and respond to changes in Mg supply in order to regulate gene expression. The Alr1 protein serves as an interesting model to investigate these regulatory mechanisms in eukaryotes. Published studies have implicated ubiquitination in the apparent post-translational regulation of Alr1-HA, and our preliminary data implicated the C-terminal domain of the Alr1 protein in its regulation by Mg supply. For this reason, it was possible that this domain is directly involved in the ubiquitination process. However, it was also possible that the use of a C-terminally epitope-tagged version of Alr1 (Alr1-HA) in experiments provided data that were not representative of the behavior of the unmodified Alr1 protein. To address these issues, my work focused on three specific aims:

I- Investigate the regulation of unmodified and N-terminally tagged Alr1 proteins.

II- If the accumulation of Alr1 is found to be post-translationally regulated, investigate mechanisms for this regulation, such as post-translational modification by ubiquitination or phosphorylation.

III- Examine the role of the potentially phosphorylated C-terminal domain in Alr1 function and regulation.

Chapter 2: Materials and Methods

2.1 Yeast and bacterial strains

2.1.1 Yeast strains

Table 2.1: Yeast strains used in this study.

Strain	Relevant genotype	Full genotype	Genetic background	Source
DY1457	Wild-type	*MATa ade6 can1-100^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-52*	W303	David Eide
NP4	*mnr2Δ*	*MATa ade6 can1-100^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-52 mnr2::KAN^{R}*	W303	Nilambari Pisat
NP10	*alr1Δ*	*MATa ade2 can1-100^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-52 alr1::HIS3*	W303	Nilambari Pisat
NP27	*alr2Δ*	*MATa can1-100^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-52 alr2::TRP1*	W303	Nilambari Pisat
NP14	*alr1Δ alr2Δ*	*MATa ade6 can1-100^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-52 alr1::HIS3 alr2::TRP1*	W303	Nilambari Pisat
NP20	*alr1Δ alr2Δ mnr2Δ*	*MATa ade2 can1-100^{oc} his3-11,15 leu2-3,112 trp1-1 ura3-52 alr1::HIS3 alr2::TRP1 mnr2::KAN^{R}*	W303	Nilambari Pisat
W3031B/D	Wild-type	*MATα ade2-1 ura3-1 his3-11 leu2-3,112 trp1-1 can1-100*	W303	R. Haguenauer-Tsapis*
MOB100	*pep4*	*MATα ade2-1 ura3-1 his3-11 leu2-3,112 trp1-1 can1-100 pep4::KanMX4*	W303	R. Haguenauer-Tsapis*
SD20	*doa4*	*MATα ade2-1 ura3-1 his3-11 leu2-3,112 trp1-1 can1-100 doa4::HIS3*	W303	R. Haguenauer-Tsapis*
SD21	*doa4 pep4*	*MATα ade2-1 ura3-1 his3-11 leu2-3,112 trp1-1 can1-100 PEP4::KanMX4 doa4::HIS3*	W303	R. Haguenauer-Tsapis*

*(Dupre & Haguenauer-Tsapis, 2001)

2.1.2 Bacterial strains

E. coli DH10β [genotype F^{-} *mcr*A Δ*(mrr-hsd*RMS-*mcrBC)* ϕ80*lacZ*ΔM15Δ *lac*X74 *rec*A1 *end*A1 *araD139* Δ(*ara*,*leu*)7697 *gal*U *gal*K λ-*rps*L *nup*G] was routinely used for cloning and propagating plasmid DNA.

2.2 Growth Media

2.2.1 Complex microbiological growth media

Luria-Bertani (LB) broth: LB was used for the routine culture of *E. coli* cells, and for antibiotic selection after transformation. LB contained 1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, and 1% NaCl; pH was adjusted to 7.0 before autoclaving. For preparation of LB plates, 1.5% agar was added before autoclaving. Where required, ampicillin was added after the medium had cooled to 45 °C, to give a final concentration of 200 μg/ml.

SOC medium: This medium was used for the outgrowth of *E. coli* following electroporation. SOC contained 2% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, 8.5 mM NaCl, 0.01 mM $MgCl_2$, and 2.5 mM KCl, adjusted to pH 7.0. A sterile solution of glucose (20 mM) was added after autoclaving.

Yeast-peptone-dextrose (YPD) medium: YPD was used for the routine growth of yeast strains. YPD contained 1% Bacto-Yeast Extract, 2% Bacto-Peptone, and 2% glucose. For culture of *alr1* and *alr1 alr2* Mg-dependent mutant strains, YPD was supplemented with 250 mM $MgCl_2$. For preparation of solid YPD medium, agar was added to a final concentration of 1.5% before autoclaving. For preparation of solid YPD medium supplemented with magnesium, solutions of 2 x YPD, 2 x agar (3%), and 5 M $MgCl_2$ were autoclaved separately, then mixed in the correct proportions to give a final 1x YPD solution containing 1.5 % agar and 250 mM Mg.

2.2.2 Minimal microbiological growth medium

2.2.2.1 Synthetic complete (SC) medium

To select for yeast strains expressing specific prototrophic markers, yeast were grown in synthetic complete (SC) medium. The complete medium contained 6.7% commercial YNB without amino acids (Q-Biogene), a carbon source (usually 2% glucose), 0.01% adenine, 0.01% L-leucine, 0.01% L-lysine, 0.01% L- histidine, 0.01% L-methionine, 0.01 % tryptophan and 0.01% uracil, and specific amino acids or bases were omitted for purposes of selection. A simplified recipe used for selection of uracil or tryptophan prototrophic strains contained 6.7% Yeast Nitrogen Base (YNB), 0.01%

Casamino acids (Difco), 0.01% uracil, 0.01% adenine, 0.01% tryptophan, and 2% glucose (uracil or tryptophan was omitted as required). For the routine culture of *alr1* and *alr1 alr2* mutant strains, $MgCl_2$ was added to SC medium to give a final concentration of 250 mM. For solid medium, 1.5 % agar was added.

2.2.2.2 Low Magnesium Medium (LMM)

For experiments where I needed to vary the Mg concentration of synthetic yeast medium, LMM was used. LMM contained 3.6% YNB without divalent cations (Q-Biogene), a carbon source (2% dextrose), divalent cations (10μM $Cucl_2$, 10μM $FeS0_4$, 10μM $MnCl_2$, 10μM $ZnCl_2$, 10mM $CaCl_2$), 0.01% adenine, 0.01 % uracil, and 0.01% of the required amino acids (usually L-tryptophan, L-leucine, L-histidine, L-lysine, and L-methionine). For selection of auxotrophic markers, nutrients were omitted as required. To avoid contamination with environmental Mg, LMM was prepared in sterile polycarbonate flasks that were pre-soaked with Citronox metal-free acidic detergent (Alconox), then rinsed twice with deionized water and twice with milli-Q water before autoclaving. LMM was prepared from sterile stock solutions and YNB powder, and filtered into a sterile polycarbonate flask. A sterile stock solution of $MgCl_2$ was added to give the Mg concentration required for the experiment.

2.3 Oligonucleotides:

All oligonucleotides were manufactured by Sigma-Genosys. Oligonucleotides were designed using ApE sequence editing software, and screened for specificity *in silicio* using Amplify 3X software prior to synthesis.

Table 2.2 Oligonucleotides used in this study.

Primer	Sequence (5'-3')	Used to construct:
myc tag 5'	TATTGCTAGAAAGCGTAAAAGCATTTTACCGGT ACCATCGATTTAAAGCTAT	YCpmyc-ALR1-HA
myc tag 3'	GTCAAGCGTAGTCTGGGACGTCGTATGGGTCAC ATCACTAATCAGTCGTA	YCpmyc-ALR1-HA
alr1-del5	GGTATTGGACCGGCACTA	YCpmyc-ALR1, YCpmyc-ALR1-Δ806, and YCpmyc-ALR1-Δ828
alr1 new HA del	ACAGCTATGACCATGATTACGCCAAGCTTTCAG TCGTAGCGGCTATA	YCpmyc-ALR1
C-term deletion	ACAGCTATGACCATGATTACGCCAAGCTTTCAT GCAGGAGGGTCTATTCT	YCpmyc-ALR1-Δ806

deletion 827	ACAGCTATGACCATGATTACGCCAAGCTTTCAG TTTCTTTTTGGCAAGAA	YCpmyc-ALR1-Δ828
K-R 5'-alr1	GTTATTTCGAGCTTCTTGCCAAAAAGAAACAGG AGATTCAATGATCGTTCCAGAAATATAAATGTA AGAGCCGGTCCTTCAAACAGATCAGTTGCAAGT C	YCpmyc-ALR1-3xK-R
Ura3-3'	CGTACCACCAAGGAATTA	YCpmyc-ALR1-3xK-R

2.4 Antibodies

Antibodies used in these studies were rabbit α-myc (Abcam, ab9106), mouse monoclonal α-HA (Abcam, ab 9110), rabbit α-Alr1 (obtained from Dr. R. Gardner), mouse monoclonal α-Vma1 (Molecular Probes), HRP-conjugated goat α-mouse (Pierce), and HRP-conjugated goat α-rabbit (Pierce).

2.5 Plasmids

All plasmids that used or generated for this study are detailed in Table 2.3.

Table 2.3: Plasmids used or generated during this study.

Plasmid	Yeast selectable marker/replication origin	Description	Source/ Reference
pFL38	URA3/CEN	Low copy yeast/*E. coli* shuttle vector	(Bonneaud et al, 1991)
YIpALR1HA	*URA3*/none	Shuttle vector containing the *ALR1* gene with a C-terminal triple HA tag	(Graschopf et al, 2001)
YCpALR1	URA3/CEN	Genomic clone containing the *ALR1* gene in pFL38	(MacDiarmid, 1997)
YCpALR1HA	URA3/CEN	pFL38 with insert of YIpALR1HA	Abhinav Pandey
pYEF2mycALR1	URA3/2μ	*ALR1* gene with *GAL1* UAS and five N-terminal MYC epitope tags.	Abhinav Pandey
YCpmycALR1HA	URA3/CEN	*ALR1* gene with native promoter, five N-terminal MYC epitope tags, and three C-terminal HA tags.	This study
YCpmycALR1	URA3/CEN	*ALR1* gene with native promoter and five N-terminal MYC tags	This study
YCpmycALR1Δ806	URA3/CEN	*ALR1* gene with native promoter, five N-terminal MYC tags and a deletion of the entire C terminal domain (from T806).	This study
YCpmycALR1Δ828	URA3/CEN	*ALR1* gene with native promoter, five N-terminal MYC tags, and a deletion of half the C-terminal domain (from K828).	This study
YCpmycALR13xK-R	URA3/CEN	ALR1 with native promoter and five N- terminal MYC tags, with Lys^{828}, Lys^{835}, and Lys^{846} mutated to arginine	This study

YCpYFPmycALR1	URA3/CEN	Alr1 with native promoter, three MYC tags and one YFP protein fused to N-terminus.	Frank Donovan, Lauren Stein
YCpYFPmycALR1Δ806	URA3/CEN	Identical to YCpYFPmycALR1 but with deletion of the entire C-terminal domain (from T806 on).	Frank Donovan, Lauren Stein
YCpYFPmycALR1Δ828	URA3/CEN	Identical to YCpYFPmycALR1 but with a deletion of half the C-terminal domain (from K828 on).	Frank Donovan, Lauren Stein

2.5.1 Plasmid construction

To construct the novel plasmids used in this work by recombination-mediated cloning, a PCR product was amplified using two oligonucleotide primers designed to add at least 32 bases of flanking DNA sequence homologous to the target plasmid to each end of the resulting DNA fragment (**Table 2.3**). Using suitable restriction enzymes, the recipient vector was then linearized between these regions of homology, combined with the PCR product, and introduced into yeast via transformation. After transformation, *URA3* prototrophic colonies were selected, and clones containing recombinant vectors were identified by the amplification of a diagnostic PCR fragment. Correct plasmids were isolated from yeast by extraction of genomic DNA for transformation of *E. coli.* Restriction enzyme mapping and DNA sequencing was used to confirm correct construction of the clones. Specific details of the construction of the new vectors are given below**.**

2.5.1.1 YCpmycALR1HA

A 5’ fragment of the *ALR1* gene (including five myc tags) was amplified from the pYEF2myc-ALR1 plasmid using the "myc tag 5’" and "myc tag 3’" oligonucleotides. The YCpALR1-HA plasmid was linearized by *Rsr*II digestion, and the PCR product was inserted via gap repair. Correct in-frame fusion of the myc tags to the HA-tagged *ALR1* gene was verified by DNA sequencing.

2.5.1.2 YCpmycALR1

A 686 bp fragment of the *ALR1* C-terminus, including the untagged C-terminal domain was amplified from genomic DNA of DY1457 using the "alr1-del5" and "alr1 new HA del" oligonucleotides. The PCR product was combined with *Msc*I and *Hind*III-cleaved DNA of YCpmycALR1HA via homologous recombination. Correct insertion of

the PCR product was verified by DNA sequencing.

2.5.1.3 YCpmycALR1Δ806

Two oligonucleotides (alr1-del 5' and C term deletion) were designed to allow amplification of the *ALR1* C-terminus (524 base pairs) from genomic DNA of DY1457, incorporating a specific deletion of the *ALR1* cytosolic C-terminal domain (Δ806). The PCR product was then combined with the YCpmyc-ALR1-HA vector that had been cleaved with *Bam*HI and *Hind*III. Correct deletion of the C-terminal domain was confirmed by DNA sequencing.

2.5.1.4 YCpmycALR1Δ828

Two oligonucleotides (alr1-del5' and del 827) were used to amplify the *ALR1* C-terminus from genomic DNA of DY1457, generating a 590 bp fragment that included a specific deletion of residues 828 to the C-terminus. The PCR product was then combined with *Msc*I and *Hind*III- cleaved YCpmycALR1HA via homologous recombination. Correct deletion of the required region of the C-terminal domain was confirmed by DNA sequencing.

2.5.1.5 YCpmycALR13xK-R

Two mutagenic oligonucleotide primers (K-R 5'-alr1 and Ura3-3') were designed to amplify a 1079 bp fragment including the *ALR1* C-terminal region from a YCpmycALR1 template. To mutate three C-terminal lysine residues (828, 835, and 846) to arginine, specific point mutations were included in the sequence of the oligonucleotide "K-R 5'-alr1". The YCpmyc-ALR1Δ828 plasmid (which includes a deletion of the targeted lysine residues) was digested with *Hind*III and *Msc*I, and the PCR product was inserted via homologous recombination. Mutation of the three lysines to arginine was confirmed by DNA sequencing.

2.6 DNA purification and manipulation

All standard methods of recombinant DNA technology, including purification of plasmid DNA from *E. coli*, restriction enzyme digestion, and the polymerase chain reaction, were performed as described previously (Franklin, 2007).

2.7 Basic yeast molecular biology

The isolation of yeast plasmid and genomic DNA was performed as described previously (Franklin, 2007). Yeast transformation was performed as described previously (Gietz et al, 1992). For strains carrying *alr1* or *doa4* mutations, care was taken to harvest cells at early log phase (0.5-0.7 A_{595}) to maximize transformation efficiency.

2.8 Protein isolation and analysis

2.8.1 Protein Extraction using trichloroacetic acid

To isolate total protein extracts from yeast strains, a 5 ml culture in the appropriate growth media was incubated at 30 °C with shaking until it reached log phase (A_{600} of 0.5-1.0). The cells were collected by centrifugation (3,000xg/5 min in a Beckman GPR centrifuge), then washed once with 10 mM EDTA (5 ml) and twice with sterile deionized water (5 ml). The cell pellet was resuspended in 1 ml of water and transferred to a 1.5 ml sterile microfuge tube. The cells were centrifuged at 13,000 rpm for 2 min in a tabletop centrifuge and the water was discarded. The pellet was resuspended in 400µl ice cold extraction buffer (10% trichloroacetic acid [TCA], 20 mM Tris-Cl pH 8.0, 50 mM ammonium acetate, 2 mM Na_2-EDTA, 2 mM PMSF, and 0.5 mM DTT) and placed on ice. Three g of glass beads were added to the cells, and the tube was vortexed for 10 min at 4°C. After the glass beads settled, the suspension containing the lysed cells were immediately transferred to a 1.5 ml microfuge tube. An aliquot of TCA extraction buffer (200 µl) was then added to the glass beads, and the mixture vortexed for an additional 2 min. The supernatant was removed and pooled with the original supernatant. The disrupted cells were collected by centrifugation (10,000x*g*/3 min), the supernatant was discarded, and 0.5 ml 100% acetone was added to the pellet. The pellet was resuspended and vortexed for 5 min to remove residual TCA, then collected by centrifugation (10,000x*g* /3 min) and dried in a Speedvac. The pellet was resuspended in 100µl buffer I (100 mM Tris base, 3% SDS, 1 mM PMSF, and 0.5 mM DTT) and boiled for 5 minutes to dissolve the protein. The suspension was centrifuged at 13,000x*g* for 5 min to pellet insoluble debris, and the supernatant containing the protein solution was transferred to a new microfuge tube and stored at -80^{o}C.

2.8.2 Measurement of protein concentration

Protein concentration was determined using a Bio-Rad DC protein assay kit, according to the manufacturer's instructions. Colorimetric reactions were quantified using an EL_x800 Universal Microplate Reader at a wavelength of 750 nm. A standard curve was constructed using BSA.

2.8.3 SDS-polyacrylamide gel electrophoresis and western blotting

All protein electrophoresis and immunnoblotting methods were performed as previously described (Franklin, 2007) except for the following modifications. To improve the sensitivity of detection, SDS-PAGE were loaded with 45-50 μg protein/lane. In experiments intended to visualize forms of Alr1 with variable gel mobility, 6-7.5 % acrylamide gels were used, and allowed to run until the tracking dye reached the bottom of the plates.

2.9 Fluorescence microscopy

Cultures of yeast cells were resuspended in 10μl of growth medium. To promote adherence of yeast cells, microscope slides were coated with 10 μl of 0.1% poly-L-lysine (Sigma), which was allowed to dry to a thin layer. Two μl of the cell suspensions were applied to the slide, followed by a cover slip, which was sealed with paraffin wax. Slides were examined using a Zeiss Axioscope fluorescence microscope and images captured using the associated software. Images were edited to remove noise, adjust contrast, and overlay frames using Photoshop CS (Adobe Systems).

Chapter 3: Results

3.1 Analysis of Alr1 accumulation

Previous work on Alr1-HA had been performed using an integrating construct (YIpAlr1-HA) obtained from the laboratory of R. Schweyen (Graschopf et al, 2001). However, this plasmid was not convenient to use because of its low transformation frequency and the difficulty of modifying it via homologous recombination. A centromeric version of this plasmid designed to overcome these drawbacks was constructed by A. Pandey (see Materials and Methods for details). To verify that the two plasmids gave a similar level of Alr1-HA accumulation, and that the use of a centromeric version did not introduce excessive variability, we compared Alr1-HA accumulation in strains transformed with the original integrated plasmid and the centromeric version. The results (**Figure 3.1A)** show that both plasmids produced a similar level of Alr1-HA accumulation in Mg -replete and deficient conditions, indicating that centromeric plasmids were suitable for subsequent experiments on Alr1 regulation.

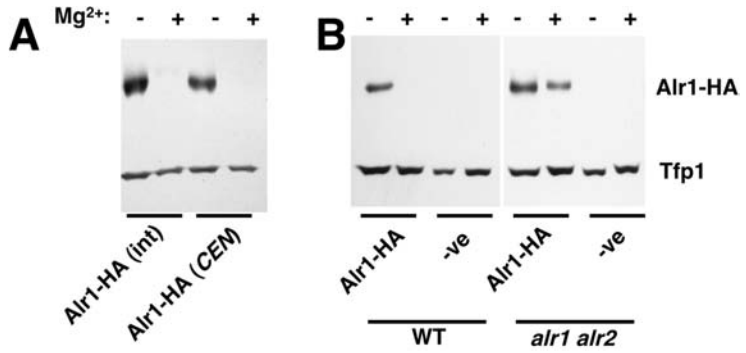

Figure 3.1 Alr1-HA accumulation. A: WT strains (DY1457) expressing C-terminally HA-tagged Alr1 from the integrative YIpALR1HA vector (int) or the centromeric YCpALR1HA vector (*CEN*) were grown in LMM-ura with 1μM (-) or 1mM Mg (+) for 16h. Protein extracts were prepared and equal amounts of protein were subjected to SDS-PAGE and immunoblotted before detection of the Alr1-HA and Tfp1 proteins. **B:** DY1457 and NP14 (*alr1 alr2)* strains transformed with YCpALR1HA were grown in LMM with 1 μM (-) or 1 mM Mg (+). Protein was extracted and fractionated by SDS-PAGE, and the Alr1-HA and Tfp1 proteins were detected by immunoblotting (-ve= untagged Alr1).

Using the centromeric version of the Alr1-HA plasmid, I then repeated an experiment performed by A. Pandey to determine the effect of *ALR* genotype on Alr1-HA protein accumulation in deficient and replete cells. HA-tagged Alr1 was expressed in WT, *alr1*, *alr2* and double mutant strains, and the effect of Mg supply on Alr1-HA accumulation was examined by immunoblotting. As shown in **Figure 3.2B**, when HA-tagged Alr1 was expressed in single mutant strains (*alr1* or *alr2*), the Alr1-HA protein accumulated to a slightly higher level in replete cells, but strongly differential accumulation was still observed. However, when this protein was expressed in an *alr1 alr2* mutant, a substantial increase in the accumulation of Alr1-HA was observed under replete conditions, which had the effect of reducing the apparent regulation of Alr1. These observations suggested that the WT Alr1 and Alr2 proteins decreased the stability of the Alr1-HA protein when these proteins were co-expressed. For this reason, I suspected that the addition of the epitope tags to Alr1 was responsible for this difference in its accumulation. Since it has been reported that the Alr1 and Alr2 proteins form homomeric and heteromeric complexes in yeast cells (Wachek et al, 2006), it is possible that complexes containing both untagged and tagged versions of Alr1 are less stable than a complex containing the HA-tagged version alone.

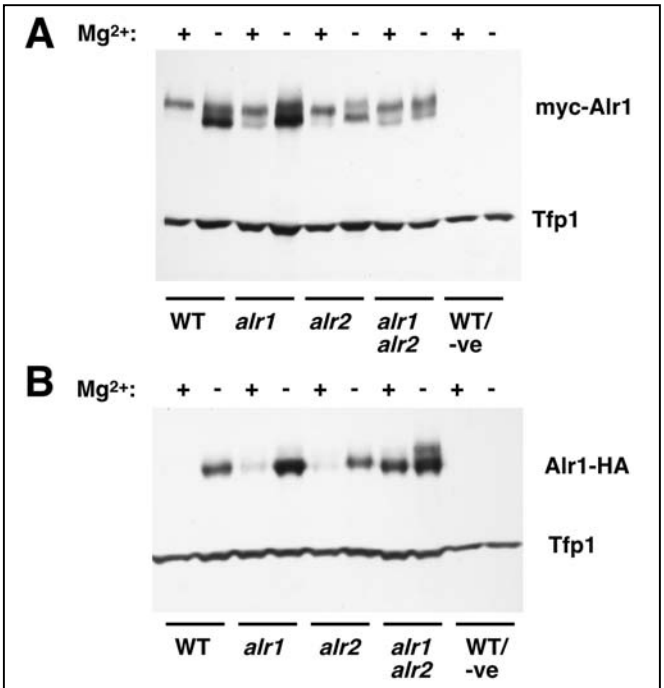

Figure 3.2 Expression of N- and C-terminally tagged Alr1 in wild-type and *alr* mutants. Strains of the indicated genotypes expressing either myc-Alr1 (A) or Alr1-HA (B) were grown for 16 h in LMM-ura containing either 1 μM (-) and 1 mM (+) Mg. Protein was extracted and fractionated by 6% SDS-PAGE, and the Alr1-HA and myc-Alr1 proteins were detected by immunoblotting (WT/-ve = untagged Alr1). Tfp1 was also detected as a loading control.

3.2 Effect of Alr1 tagging on accumulation

The above observations suggested that the modification of the C-terminal domain of Alr1 influenced the stability of the protein, and implied that this end was sensitive to modification. Since the modification of Alr1 by addition of tags to other domains was important for later analysis of its regulation, I tested if the addition of tags to the N-terminal end also had an effect on the behavior of the protein. For these experiments, I used a version of Alr1 with five N-terminal myc tags, expressed from the native *ALR1* promoter (see **Chapter 2.6.2** for details of plasmid construction). The function of this version was confirmed by complementation of the growth defect of an *alr1 alr2* mutant **(Figure 3.8A)**. The construct was introduced into WT, *alr1, alr2* and *alr1 alr2* mutant strains, and the resulting strains were grown in LMM supplied with 1 μM and 1 mM Mg. Alr1 expression was determined by immunoblotting of total protein extracts. As illustrated in **Figure 3.2A**, expression of this protein in a WT strain gave different results from those observed with Alr1-HA. Although accumulation of the myc-Alr1 protein was elevated in deficient conditions by approximately 2-fold, the difference in accumulation was much less than was observed for Alr1-HA. Furthermore, expression of myc-Alr1 in the three mutant strains had no, or only a minor effect on the relative abundance of the protein in Mg-deficient and replete conditions. As for Alr1-HA, accumulation of myc-Alr1 in the *alr1 alr2* mutant was similar in both conditions. Thus, N-terminally tagged Alr1 displayed a different response to strain genotype and Mg supply as the Alr1-HA protein.

To further investigate if the effect of Mg supply on Alr1-HA accumulation was an artifact of modifying the protein, I examined the accumulation of myc-tagged and unmodified forms of Alr1 in strains supplied with varying Mg concentrations (**Figure 3.3**). Alr1 was detected by using an α-Alr1 antibody raised against the C-terminal cytosolic domain (a gift of R. Gardner). Use of the anti-Alr1 antibody alone to probe a protein extract from an *alr1 alr2* strain expressing unmodified Alr1 generated two cross-reacting bands (**Figure 3.3A**). Based on the apparent molecular weight of the bands, I identified the higher band as Alr1, indicating that the lower band probably represented a non-specific cross-reaction with an unrelated protein. This assignment was confirmed by analysis of a

non-complemented *alr1 alr2* strain (**Figure 3.3B**, *alr1 alr2* + V lane). The higher molecular weight band was absent in protein from this strain, while the lower molecular weight band remained (indicated by an asterisk). To further examine antibody specificity, I constructed a version of Alr1 with a deletion of the C-terminal cytosolic domain (Alr1-Δ806) (see **Chapter 2.6.2** for details of plasmid construction). This protein (which is described in more detail later in this work) was not detected by the Alr1 antibody, confirming the specificity of antibody for the C-terminal domain (**Figure 3.3B**). Finally, when Alr1 was expressed in an *alr1* single mutant, the high molecular weight band was detected with the α-Alr1 antibody, but in a non-complemented control *alr1* strain, this band was absent (**Figure 3.3C**, V lane). This observation indicates that the α-Alr1 antibody did not detect the Alr2 protein. The specificity of this antibody for Alr1 may be a consequence of sequence differences between the two proteins in the C-terminal domain (data not shown), or it could indicate that Alr2 is expressed at levels too low for the Alr1 antibody to detect. In either case, the antibody allowed me to specifically detect the Alr1 protein in these strains.

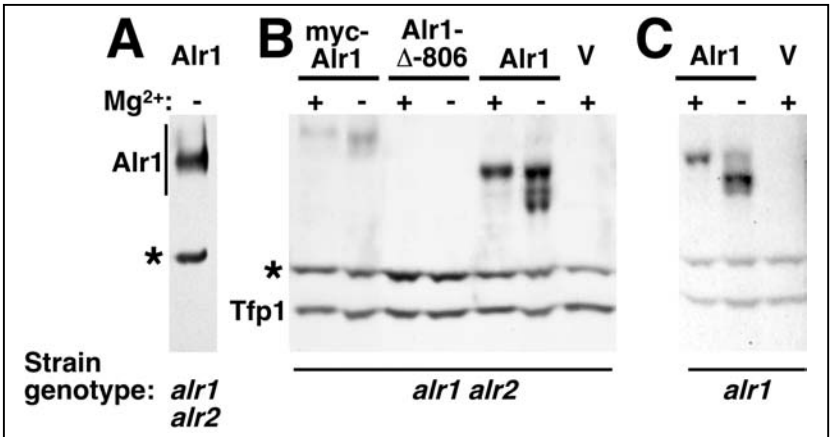

Figure 3.3 Accumulation of untagged and N-terminal tagged Alr1 (**A**) An *alr1 alr2* strain (NP14) transformed with YCpAlr1 was grown in LMM-ura supplied with 1 μM Mg (-). Protein was extracted and fractionated by SDS-PAGE (6% acrylamide), and the gel blotted to nitrocellulose. The blot was probed with an α-Alr1 antibody. The identity of the bands is indicated based on comparison to molecular weight standards (the asterisk indicates a non-specific band detected by the anti-Alr1 antibody). (**B**) An *alr1 alr2* strain (NP14) was transformed with YCpmyc-Alr1 (myc-Alr1), YCpmycAlr1Δ806 (Alr1Δ806), YCpAlr1 (Alr1), or pFL38 (V). V-only control was grown in the LMM+250mM Mg and the other resulting strains were grown in LMM containing 1 μM (-) or 1 mM (+) Mg. Protein extracts were analyzed by immunoblotting with α-Alr1 and α-Tfp1 antibodies (as a loading control). (**C**) An *alr1* mutant (NP10) was transformed with YCpAlr1 (Alr1) or pFL38 (V). The strains grown in LMM supplemented with Mg as indicated and Alr1 and Tfp1 detected as described in (**B**).

untagged Alr1 was expressed in either an *alr1* or *alr1 alr2* mutant, a small but reproducible effect of Mg supply on abundance was observed (**Figure 3.3B, C**). Expression of myc-tagged Alr1 in the *alr1 alr2* mutant showed that, while the myc-tagged version accumulated to a lower overall level compared with the untagged version, there was only a minor effect of Mg supply on the relative abundance of the protein. These observations suggested that the myc-tagged version of Alr1 behaved similarly to the untagged version in its response to Mg supply. The difference in the overall level of the myc-tagged and native Alr1 proteins may represent a consequence of the difference in the basic vector backbone used, or it may result from differences in the efficiency with which each protein is translated, or their post-translational stability.

3.3 Genetic analysis of Alr1 accumulation

3.3.1 Effect of *doa4* and *pep4* mutations on Alr1 accumulation

A previous study reported that factors required for the post-translational regulation of membrane proteins affected Alr1-HA abundance and stability (Graschopf et al, 2001). To further examine the effect of epitope-tagging Alr1 on its apparent stability, a previous student in this laboratory (A. Pandey) examined the effect of inactivating specific regulatory factors on the abundance of the HA-tagged version of Alr1. The results of this work indicated that the accumulation of Alr1-HA in Mg-replete cells was strongly increased by mutations in factors required for post-translational regulation, including *rsp5* (*npi1*), *pep4*, and *doa4*. The *PEP4* gene is required for the maturation of vacuolar proteases essential for the degradation of proteins delivered to the vacuole lumen (Jones et al., 1982), and Alr1-HA accumulated to a similar high level in *pep4* cells grown in high and low-Mg conditions (A. Pandey and C. MacDiarmid, unpublished data). This observation suggested that in Mg-replete conditions, Alr1-HA was delivered to the vacuole lumen and degraded via a Pep4-dependent mechanism. The Rsp5 E3 ubiquitin (Ub) ligase covalently links Ub to membrane proteins at the PM and Golgi (Hein et al, 1995), and was previously reported to be required for the down-regulation of Alr1-HA in response to Mg repletion (Graschopf et al, 2001). The *npi1* mutation, which reduces the expression of Rsp5 by 90% (Hein et al, 1995), also substantially increased Alr1-HA accumulation in both Mg-replete and deficient conditions. Doa4 is a Ub-

hydrolase that removes Ub from membrane proteins before they are packaged into multi-vesicular bodies (MVB's) at the late endosome (Amerik et al, 2000; Swaminathan et al, 1999). *doa4* mutants are unable to remove Ub from ubiquitinated membrane proteins in the PVC, and are consequently unable to sort them into the lumen of the MVB (Nikko & Andre, 2007), increasing the stability of these proteins in non-permissive conditions. A *doa4* mutant showed strongly enhanced accumulation of Alr1-HA (A. Pandey and C. MacDiarmid, unpublished data). Lastly, the effect of mutating the *END3* gene, which is required for the endocytosis of plasma membrane proteins (Raths et al, 1993) was also examined. The *end3* mutation did not affect the steady-state level of Alr1-HA accumulation, suggesting that endocytosis of Alr1-HA was not required for its degradation (A. Pandey and C. MacDiarmid, unpublished data).

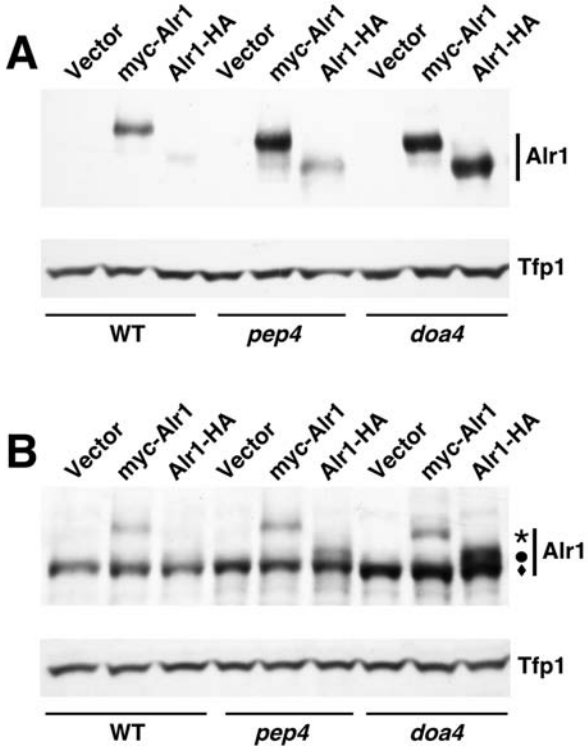

Figure 3.4 Modification of the C-terminus affects Alr1 accumulation. (A) Strains of the indicated genotypes (W303/WT, MOB100/*pep4* and SD20/*doa4*) expressing Alr1-HA or myc-Alr1 were grown for 16 h in LMM-ura with 1 mM Mg, protein extracted, and 40 µg of each sample fractionated on 6% polyacrylamide gels. After blotting to nitrocellulose, Alr1 variants were detected with anti-HA (A) and anti-myc (A), or anti-Alr1 (B) antibodies. In (B), the positions of bands corresponding to myc-Alr1 and Alr1-HA are indicated by the * and | symbols respectively, and the t symbol indicates unmodified Alr1. Tfp1 was detected as a loading control.

To determine the role of these factors in regulating the abundance of the unmodified form of Alr1, and to compare the relative abundance of the modified and unmodified forms of Alr1, I determined the effect of the *doa4* and *pep4* mutations on the accumulation of the HA, myc and untagged versions in cells grown in "non-permissive" (Mg-replete) conditions. These two mutations were chosen because previous experiments indicated that they had the largest effect on Alr1-HA accumulation under replete conditions (A. Pandey and C. MacDiarmid, unpublished

data). Since the *pep4* mutation stabilizes proteins delivered to the vacuole, and the *doa4* protein prevents delivery of ubiquitinated proteins to the vacuole lumen, these mutations were expected to provide information on the degree to which the three forms of Alr1 were substrates for Ub-mediated degradation pathways. Initially, I detected the tagged forms of Alr1 using antibodies to the epitope tags (**Figure 3.4A**). The results of this experiment confirmed that the two tagged versions of Alr1 behaved differently. In this experiment, the *pep4* and *doa4* mutations increased accumulation of the Alr1-HA protein by 3 and 6-fold respectively (as determined by densitometry), but had a smaller effect on the myc-Alr1 and native proteins (a < 2 fold increase). The effect of the *pep4* mutation in this experiment was somewhat smaller than seen in previous experiments (data not shown). This variation may be a consequence of an effect of the *pep4* mutation on the reliability of Alr1 recovery in protein extracts (data not shown).

The same set of protein samples were then subjected to Western Blot analysis using an α-Alr1 antibody to detect all three forms of Alr1 (**Figure 3.4B**). Due to differences in gel mobility, the three versions of Alr1 could be clearly discriminated as indicated. These data illustrate that: i) the expression of the native Alr1 was relatively unaffected by the two mutations, although *doa4* increased its accumulation approximately 2-fold; ii) the Alr1-HA form was expressed at a substantially lower level than the native protein in the WT strain, but was substantially stabilized by the *pep4* and *doa4* mutations; and iii), the myc-tagged Alr1 protein accumulated to an approximately 2-fold lower level than the native protein (consistent with **Figure 3.3B**), but responded to the two mutations to a similar degree as the native version. Thus, a strong influence of factors required for post-translational regulation and ubiquitin-mediated degradation on Alr1 accumulation was restricted to the C-terminally tagged Alr1 protein. The untagged and N-terminally tagged versions of Alr1 were both much less responsive to Mg supply, and their accumulation was less dependent on factors required for post-translational regulation.

3.3.2 Effect of *alr2* and *mnr2* mutations on Alr1 accumulation

The observation that the Alr1-HA protein was not representative of the behavior of the untagged protein led me to reexamine the influence of other mutations on Alr1

abundance. In particular, I wanted to determine the effect of *mnr2* and *alr2* mutations on the accumulation of the native Alr1 protein. The *mnr2* mutation was previously shown to increase the accumulation of Alr1-HA (Pisat, 2009), and the *alr2* mutation also slightly increased Alr1-HA accumulation (**Figure. 3.2**B and data not shown), although its effect has not been examined in detail. Since it was previously believed that the effect of the *mnr2* mutation was evidence for a process of Alr1 regulation, I wanted to determine the effect of the mutation on the accumulation of unaltered Alr1.

To perform this experiment, WT, *alr2* and *mnr2* strains were grown over a range of Mg concentrations, and the native Alr1 protein was detected using the α-Alr1 antibody **(Figure 3.5)**. The response of untagged Alr1 to varying the Mg supply was noticeably different from the HA-tagged version. In samples from WT replete cells (supplied with 100 and 1000 μM Mg), Alr1 was detected predominantly as a single band, while an additional faster-migrating band was also observed in protein from deficient cells (1 and 10 μM Mg). This variation in mobility was also observed in a previous study using Alr1-HA (Graschopf et al, 2001). The authors suggested that these two bands may represent differentially phosphorylated forms. The appearance of this new form of Alr1 in deficient cells increased the overall accumulation of the protein by approximately 100%. This observation was in strong contrast to previous observations of a 16-fold difference between Alr1-HA abundance in deficient and replete cells (Pisat, 2009), and strongly indicates that Mg supply has relatively little effect on the abundance of the unmodified Alr1 protein.

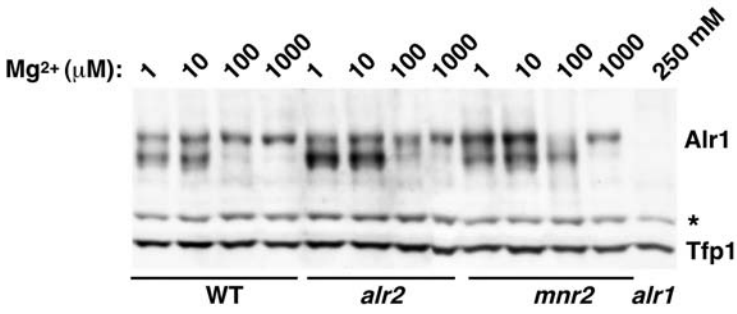

Figure 3.5 Effect of transporter mutations on Alr1 accumulation. WT (DY1457), *alr2* (NP27) and *mnr2* (NP4) strains were grown to exponential phrase in LMM supplemented with the indicated concentration of Mg. Protein was extracted and equal amount of protein (50 μg) was fractionated by 6% SDS-PAGE and blotted to nitrocellulose. The Alr1 and Tfp1 proteins were detected by anti-Alr1 and anti-Tfp1 antibodies. As a negative control for Alr1 antibody specificity, protein was extracted from *alr1* (NP10) mutant cells that were grown in SD medium with 250 mM Mg for 16 h. Non-specific bands generated by the anti-Alr1 antibody are indicated by an asterisk.

Both the *alr2* and *mnr2* mutations were associated with a small increase in the

accumulation of Alr1 under Mg-deficient conditions, but had little effect in relatively replete conditions (100-1000 μM Mg). In particular, a 6-fold increase in Alr1 accumulation previously seen in the *mnr2* mutant expressing Alr1-HA when supplied with 100 μM Mg (Pisat, 2009) was not observed for the native protein. These results again demonstrated that the Alr1-HA protein does not accurately represent the behavior or regulation of the native form, and that the effect of perturbing Mg homeostasis on Alr1 abundance is relatively minor.

3.4 Effect of Mg supply on Alr protein activity

My observations that the accumulation of the untagged Alr1 protein was relatively insensitive to Mg supply led others in the laboratory to re-examine the idea that Mg transporter activity was increased by Mg deficiency in yeast. Some indirect evidence for this regulation has been reported. For example, genetic changes thought to alter Mg homeostasis had effects consistent with changes in Alr system activity. Characterization of the vacuolar Mg transporter Mnr2 established that the *mnr2* mutation reduced tolerance to several divalent cations, while also increasing their accumulation by yeast cells (Pisat, 2009; Pisat et al, 2009). The latter effect was

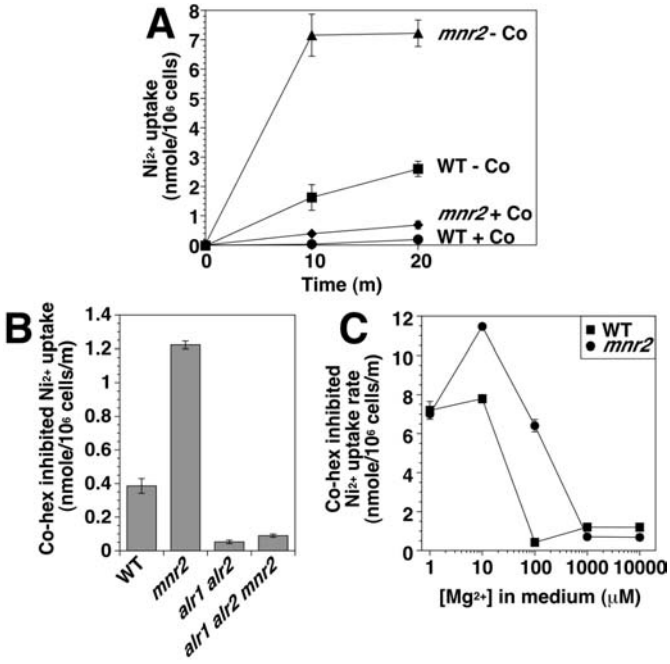

Figure 3.6 Co(III)-hexaamine-sensitive Ni^{2+} uptake in yeast. (**A**) Time course of Ni^{2+} uptake by WT (DY1457) and *mnr2* (NP4) strains. Cells were added to uptake buffer containing 100 μM Ni^{2+}, with or without Co(III)-hexaamine chloride (Cohex) (100 μM). At the indicated times, aliquots were removed and processed for measurement of Ni^{2+} content by ICP-MS. (**B**) Rate of Cohex-inhibited Ni^{2+} uptake by WT (DY1457), *mnr2* (NP4), *alr1 alr2* (NP14) and *alr1 alr2 mnr2* (NP20) yeast strains. Ni^{2+} uptake was determined with +/- 100 μM Cohex 5 min after addition of 100 μM Ni^{2+}. (**C**) Cohex-sensitive Ni^{2+} uptake activity is responsive to Mg supply. DY1457 (WT) and *mnr2* (NP4) strains were grown for 6 h in LMM with the indicated Mg concentration, and the initial rate (1 min) of Ni^{2+} uptake was determined. Values indicate Cohex-sensitive activity. (All experiments were performed by N. Gadhia).

exacerbated by growth of the mutant strain in Mg-deficient conditions. These phenotypes might be attributed to an increase in the expression of a non-specific divalent cation transporter in the *mnr2* mutant, and the Alr proteins are good candidates for this system. Overexpression of Alr1 or Alr2 was previously reported to increase divalent cation sensitivity, and to increase Co^{2+} uptake (MacDiarmid & Gardner, 1998b), which is consistent with an ability of the Alr systems to mediate the uptake of divalent cations other than Mg.

To directly test the response of the Alr systems to Mg supply, others in my laboratory used Ni^{2+} as a tracer to follow the activity of the Alr systems in cells supplied with different Mg concentrations, or with mutations in Mg transport systems (N. Gadhia and C. MacDiarmid, unpublished data, **Figure 3.6**). Ni^{2+} ion was used as a surrogate for Mg in order to overcome some of the technical limitations of available methods to measure Mg, such as AAS and ICP-MS. These methods are not accurate enough to measure small changes in the total Mg content of yeast cells, which depending on growth conditions, can contain large amounts of Mg. Evidence that Ni^{2+} transport by yeast is mediated primarily via the Alr proteins comes from the use of Co(III)-hexaamine, a specific inhibitor of the Alr systems (**Figure 3.6A**), and also by the observation that mutations which inactivated the Alr systems substantially reduced Co(III)hexaamine-sensitive Ni-uptake activity (**Figure 3.6B**). In summary, these experiments demonstrated that reducing yeast Mg supply substantially increased the activity of the Alr systems, providing direct evidence for regulation. Activity was observed to increase 16-fold when wild-type cells were grown in 10 μM Mg compared to 1 mM or higher concentrations (**Figure 3.6C**). In addition, a 12-fold increase in Alr system activity was observed in *mnr2* cells grown in synthetic medium with 100 μM Mg (**Figure 3.6C**), indicating that genetic methods to reduce the cytosolic Mg supply also increased Alr system activity. Thus, these experiments confirmed that yeast cells compensate for changes in intracellular Mg supply by regulating the activity of the Alr systems. However, my observations indicate that these changes are not likely to be achieved via the control of Alr1 gene expression. For example, I observed very little difference in Alr1 protein accumulation in WT and *mnr2* cells supplied with 100 μM Mg (**Figure 3.5**), despite the large effect that the *mnr2* mutation had on Alr system activity under these conditions

(**Figure 3.6C**).

3.5 Genetic investigation of Alr1 C-terminal domain function

The relatively small changes in Alr1 protein abundance with Mg supply that I observed in my experiments suggested that I examine other possible mechanisms for the regulation of Alr1 activity. I decided to investigate the role of post-translational modification of Alr1. My experiments (**Figure 3.5**), as well as data from other workers in this laboratory (Pisat, 2009) and previous studies (Graschopf et al, 2001; Wachek et al, 2006) indicated that the Alr1 protein was modified post-translationally in response to changes in Mg supply. Because the form of Alr1 with lower mobility in SDS-PAGE was

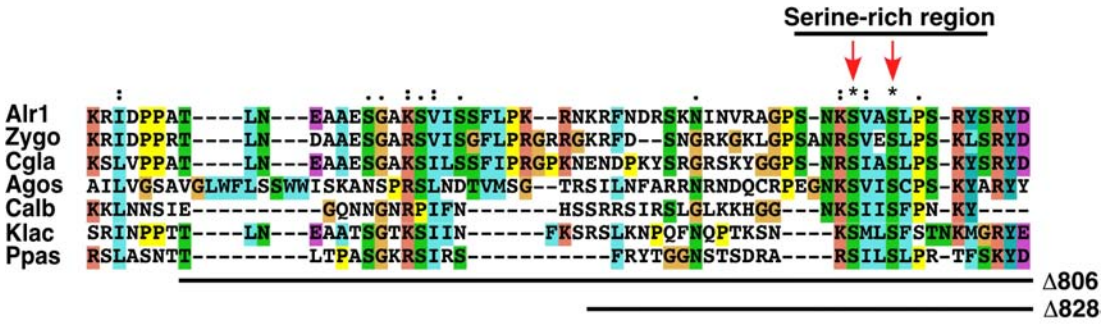

Figure 3.7 Sequence alignment of the C-terminal domain of Alr1 homologs. The location of two conserved serine residues (S847 and S850) identified as phosphorylated (Phosphopep database, Bodenmiller et al 2008) are indicated by red arrows. The extent of the deletions constructed in this region of Alr1 are shown as black lines (residue numbers indicate the position of the first residue deleted in the native Alr1 sequence). Sequences included in the lineup are from *S. cerevisiae* S288C (Alr1, Genbank # EDN63746), *Zygosaccharomyces rouxii* (Zygo, NCBI XP_002495778), *Candida glabrata* (Cgla, NCBI XP_445759), *Ashbya gossypii* (Agos, NCBI NP_983925), *Candida albicans* (Calb, Genbank EEQ44297), *Kluyveromyces lactis* (Klac, NCBI XP_454276), and *Pischia pastoris* (Ppas, NCBI XP_002490207).

partially converted to a higher-mobility form by treatment with lambda phosphatase, a previous study concluded that this modification was phosphorylation (Wachek et al, 2006). In experiments performed in this laboratory (Pisat, 2009), when replete cells were transferred to Mg-free medium, the rate at which the Alr1 protein was modified from a lower mobility to a higher mobility form depended on the intracellular Mg supply, as cells loaded with Mg prior to transfer showed a slower rate of this transition. In addition, the *mnr2* mutation substantially increased the speed of this transition, consistent with this mutant having reduced access to intracellular Mg stores. Thus, modification of the Alr1 protein appears to be responsive to Mg supply.

To further investigate this connection, I searched for residues implicated as targets

for phosphorylation in Alr1. Several groups have characterized the yeast phosphoproteome by using mass spectrometry (Ficarro et al, 2002). The results of these studies have been collated into web databases such as PeptideAtlas (King et al, 2006) and Phosphopep (Bodenmiller et al, 2007), providing a resource of information on sites of yeast protein phosphorylation. Searching this database for phosphorylated residues in Alr1 revealed five candidate residues, including two serine residues (S847 and S850) located in the C-terminal cytosolic domain. These two residues were of interest because both are tightly conserved between closely related Alr1 homologs (they are identical in the 7 fungal Alr1 homologs shown) (**Figure 3.7**). The existence of these tightly conserved residues at the C-terminal end of Alr1 suggested that they might play a role in regulating Alr1 activity.

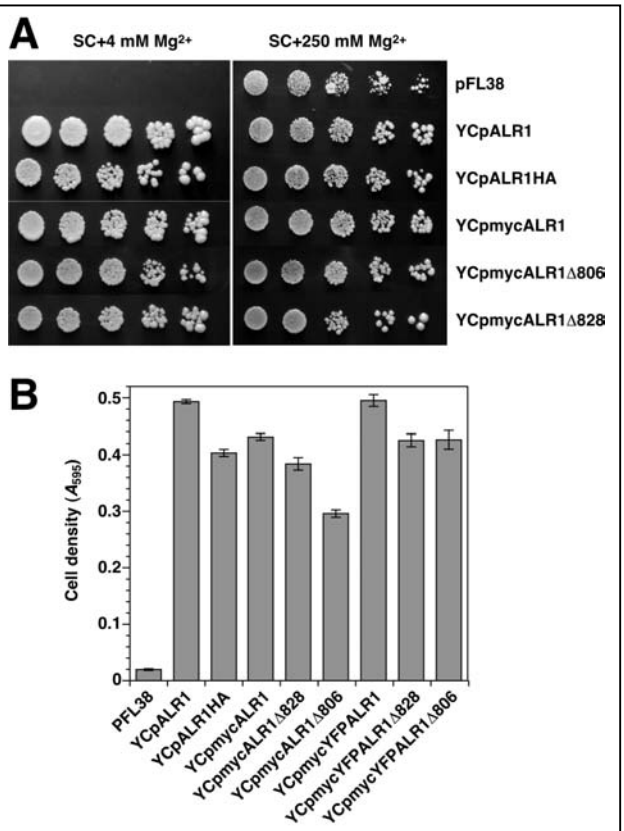

Figure 3.8 Effect of Alr1 modifications on function. **A**) Cultures of *alr1 alr2* (NP14) strains carrying the indicated constructs were grown to saturation in SC-ura medium with 4 mM Mg. A five-fold serial dilution series was prepared, and equal volumes of cells applied to SC-ura medium containing 4 mM Mg (left panel) or 250 mM Mg (right panel). Plates were incubated at 30° C for 3 days before photography. **B**) Strains from A, and additional NP14 transformants carrying the YFP-tagged Alr1 plasmids indicated, were grown in high Mg medium (SC+ 250 mM Mg), then used to inoculate aliquots of LMM + 30 μM Mg to give an initial A_{595} of 0.01. Cultures were grown for 16 h before recording final A_{595} values (values are means of three replicate cultures, error bars = +/- 1 SEM).

To test the function of this region, I made two deletions of the C-terminal domain. One deletion removed all residues from lysine 828 to the C-terminus, and another removed all residues from threonine 806 to the C-terminus. These deletions were primarily chosen to determine the effect of removing the C-terminal serine-rich region containing the two conserved serine residues (Δ828) compared with the entire C-terminal region (Δ806), which contains other less

well-conserved serine residues that are also potential targets for post-translational modification. Since the deletion of the C-terminal domain removed the residues recognized by the α-Alr1 antibody, the deletions were introduced into an Alr1 construct that included several N-terminal myc tags, enabling detection of the modified forms by Western blotting. Other workers in the laboratory (L. Stein and F. Donovan) then constructed derivative plasmids that contained the YFP gene fused directly to the N-terminal end of Alr1, Alr1Δ828, and Alr1Δ806 (these constructs also included the myc tags at the N-terminus of the YFP ORF), for the purpose of determining the effect of the deletions on Alr1 location (see **Chapter 2.6.2** for details of plasmid construction).

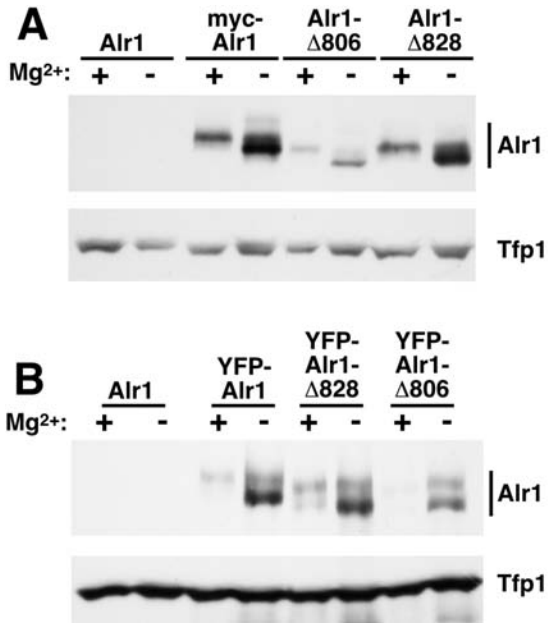

Figure 3.9 Effect of C terminal domain deletions on Alr1 stability. A) A WT strain (DY1457) was transformed with YCpAlr1 (Alr1), YCpmyc-Alr1 (myc-Alr1), YCpmyc-Alr1Δ806 (Alr1Δ806), and YCpmyc-Alr1Δ828 (Alr1Δ828) constructs, and the strains grown in LMM-ura containing 1 μM (-) or 1 mM (+) Mg. Total protein extracts were prepared and 50 μg of protein was fractionated by SDS-PAGE and transferred to nitrocellulose. Myc-tagged proteins were detected by immunoblotting of total protein extracts with an anti-myc antibody. Protein from a strain expressing untagged Alr1 was included as a control for antibody specificity. Tfp1 was detected as a loading control. **B**) An *alr1* mutant (NP10) was transformed with YCpAlr1, YCpmycYFPAlr1, and two YFP-tagged C-terminal deletion plasmids (as describe in **Figure 3.8**). Western blotting was performed and the Alr1 and Tfp1 proteins detected as described in A.

I first determined if these novel versions of Alr1 were functional and stable (**Figures 3.8A and 3.8B**). Both myc-tagged deletions effectively complemented the growth defect of an *alr1 alr2* mutant in medium with replete (4 mM, **Figure 3.8A**) or suboptimal (30 μM, **Figure 3.8B**) Mg concentrations. However, deletion of the entire C-terminal domain in the myc-tagged version was associated with a minor growth defect in suboptimal Mg (**Figure 3.8B**), suggesting that this version might not be entirely functional. However, the function of this deletion was not severely affected, as a major effect on growth was not observed when the same deletion was introduced into a YFP-tagged version (YCpmycYFPAlr1Δ806, **Figure 3.8B**). Thus in general, modifications to the C-terminal

domain do not appear to substantially affect the ability of Alr1 to perform its basic function of Mg transport.

I next determined the effect of the deletions on Alr1 protein accumulation in yeast cells (**Figure 3.9**). I measured the abundance of both the myc-tagged versions of the proteins (**Figure 3.9A**) and the YFP-tagged versions (**Figure 3.9B**), after growth in both low and high Mg concentrations. In general, the YFP and myc-tagged versions of these proteins accumulated to detectable levels, and both showed approximately 3-fold elevated accumulation in deficient cells. Proteins with the Δ828 accumulated similarly to the corresponding full-length versions, indicating that this deletion had little effect on abundance. However, deletion of the entire C-terminal domain was associated with a decrease in protein accumulation for both myc and YFP-tagged proteins, indicating that this modification may alter the production or stability of Alr1.

3.6 Effect of Alr1 deletions on subcellular location

The western blotting experiments described above had revealed that deletion of the entire C-terminal domain of Alr1 may partially reduce its function, at least for some versions of the protein. To determine if this effect was associated with a change in the subcellular location of the protein, I determined the location of the three YFP-tagged constructs in complemented *alr1* strains grown under replete and deficient conditions. As shown in **Figure 3.10** (Alr1), control cells that expressed untagged Alr1showed a low level of diffuse autofluorescence. In the same strain expressing YFP-Alr1, a low level of accumulation was observed in both Mg-deficient and replete cells. This result indicates that despite the small change in protein abundance under replete conditions, there was little apparent effect on the location of the protein in yeast cells. When compared with the control (Alr1) cells, the YFP-Alr1 signal was apparent as a punctate signal associated with the plasma membrane. A small increase in this signal was observed in deficient cells, but the relative distribution of the signal in the cells did not change. When I examined cells expressing modified versions of Alr1, some differences were observed. Compared to replete cells expressing YFP-Alr1, cells expressing the Δ806 and Δ828 versions of Alr1 showed a somewhat brighter YFP signal. The punctate distribution of the signal at the cell surface was retained, but there was somewhat more signal observed

within the cell. Comparison with the DIC images indicated that this signal was apparently located within the vacuole lumen. No substantial difference was observed between the two deletions in the intensity of this vacuolar YFP signal.

In cells expressing YFP-Δ828 and YFP-Δ806 under Mg-deficient conditions, the distribution of YFP signal was altered from that seen in replete cells. Somewhat less signal was observed at the cell surface, and more protein was seen in a location consistent with its accumulation in the lumen of the vacuole. At present, these observations are not conclusive (only two independent experiments were performed, and some variation in the YFP signal intensity and location was observed). In particular, I cannot be sure that the signal observed in the lumen of the vacuole represents the entire protein, or simply results from the enhanced accumulation of the relatively protease resistant YFP domain (the absence of partially degraded forms of Alr1 in western blotting experiments would appear to suggest that intact or partially degraded Alr1 does not normally accumulate in the vacuole lumen). However, at present my observations suggest that modification of the C-terminal domain (and in particular, the deletion of the last half of this domain) had a minor effect on the abundance and location of the Alr1 protein, and that the proteins were still able to accumulate to detectable levels in the plasma membrane. These results also strongly indicate that Mg status does not have a large effect on the distribution or abundance of the Alr1 protein, a finding which

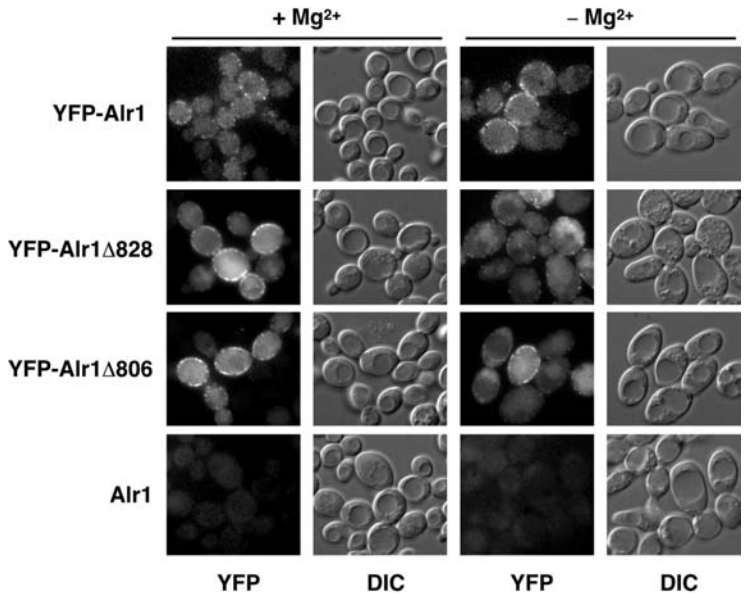

Figure 3.10 Subcellular location of YFP-tagged Alr1 and derivatives. An *alr1* mutant (NP10) was transformed with the indicated constructs. The resulting strains were grown to exponential phase in SC-ura, then transferred to LMM-ura containing 5μM (-) or 5 mM (+) Mg and grown for an additional six hours. Live cells were examined by epifluorescence microscopy. Fluorescence images (YFP) and corresponding differential interference contrast images (DIC) are shown. Images are representative of two independent experiments.

again contrasts with a previous report of Alr1 regulation (Graschopf et al, 2001).

3.7 Discussion

The CorA family of Mg channels is widespread in biology, and members play an important role in the regulation of cytosolic Mg concentration. Although the expression of most characterized CorA proteins is constitutive, a previous study (Graschopf et al, 2001) suggested that the expression of the major Mg uptake system Alr1 was regulated by Mg supply. If so, this regulation might contribute to more efficient uptake of Mg under deficient conditions, as has been shown to occur for other metal ion transporters. My data contradict some aspects of this model, while refining other aspects.

3.7.1 Effect of Mnr2 on Alr system activity

In addition to its role in Mg homeostasis, the *mnr2* mutation was also associated with changes in content of other elements, including divalent cations (Pisat et al, 2009). For example, *mnr2* mutants were more sensitive to Mn^{2+}, Ca^{2+} and Zn^{2+}, and overaccumulated these metals when grown in Mg-deficient conditions (Pisat et al, 2009). These observations suggested that a low-affinity divalent cation transport system was upregulated in the *mnr2* mutant (Pisat et al, 2009). Since the Alr proteins mediate relatively non-specific divalent cation uptake (MacDiarmid & Gardner, 1998a), it was suspected that the *mnr2* mutation increased the activity of these systems (Pisat et al, 2009). Studies performed by others in this laboratory appear to support this model. Both Mg-deficient yeast cells and *mnr2* mutant strains exhibited increased activity of a cobalt(III)-hexaamine sensitive Ni^{2+} uptake system, indicating the involvement of a CorA family transport system (**Figure 3.6**). When the *mnr2* mutation was combined with the *alr1* and *alr2* mutations, the increase in activity was suppressed, indicating that elevated Ni^{2+} uptake was primarily attributable to the Alr systems, rather than a novel independent Ni^{2+} uptake system.

Previous workers in this laboratory had established that *ALR1* gene expression was not regulated by Mg supply (A. Achari and C.MacDiarmid, unpublished results), and that the *mnr2* mutation had no effect on *ALR1* promoter activity. Both observations are inconsistent with a model in which Alr system activity is increased as a consequence of

Mg-responsive gene expression. For this reason, the goal of my work was to investigate potential mechanisms of Alr1 regulation by Mg supply, and specifically to determine the mechanism of the apparent post-translational regulation of Alr1 protein abundance. Previous work had shown that Alr1-HA stability was affected by Mg supply (A. Pandey and C. MacDiarmid, unpublished data). Consistent with this model, I also observed a large change in Alr1-HA accumulation in Mg-deficient and replete conditions **(Figure 3.1, 3.2B)**. However, in my experiments, a much less pronounced effect of Mg supply on the accumulation of the native (untagged) protein was observed **(Figure 3.3C, 3.4)**. A direct comparison of the level of Alr1 protein and Alr1-HA that accumulated in cells expressing both forms indicated that the HA tagged form was much abundant than the native form in Mg-replete cells, suggesting that the tag itself was responsible for the majority of the apparent "regulation" of Alr1 accumulation **(Figure 3.4)**. In addition, while the *mnr2* mutation had a large effect on the accumulation of HA-tagged Alr1 (Pisat et al, 2009), this mutation had much less effect on the accumulation of the untagged protein. These observations are consistent with a model in which the HA tag destabilized the Alr1 protein in replete conditions, creating the artifactual appearance of regulation. A difference in native protein accumulation does not explain the Mg-regulated transport activity that we observed, as indicated by the observation that growth of WT cells in 10 μM vs 1 mM Mg produced a substantial increase in Alr system activity **(Figure 3.6C)**, but only a 2-fold change in the accumulation of the native Alr1 protein **(Figure 3.5)**. In addition, the inactivation of factors important for the Ub-dependent regulation of protein stability (Doa4 and Pep4), which had substantial effects on the relative accumulation of Alr1-HA in deficient and replete conditions (A. Pandey and C. MacDiarmid, unpublished data), had much less effect on the native protein and other N-terminally tagged versions (**Figure 3.4**). A key observation was that mutations that eliminate endocytosis (*end3* and *dim1*) had no effect on relative Alr1-HA accumulation in deficient vs replete conditions (A. Pandey and C. MacDiarmid, unpublished data), which is inconsistent with the regulation of Alr1 accumulation by control of its internalization rate.

Based on these observations, I suggest that Alr1-HA abundance may primarily be determined by the regulated sorting of Alr1 prior to its arrival at the cell surface. Perhaps

as a consequence of misfolding, HA-tagged Alr1 may be detected by protein quality control mechanisms in the ER or late Golgi and sorted to the vacuole for degradation. The observation that *npi1*, *doa4* and *pep4* mutations stabilize Alr1-HA in replete conditions suggests that this protein is not a target for the ERAD pathway, since ERAD does not depend on Rsp5 or Doa4 activity. I suggest that this quality control process primarily occurs in the late Golgi, which is known to be a site for Rsp5 activity. In contrast, the native protein and N-terminally tagged versions were apparently not subject to ubiquitin-dependent quality control mechanisms, since their accumulation was not substantially increased in *pep4* and *doa4* mutants **(Figure 3.4)**. Finally, the effect of Mg supply and the *mnr2* mutation on Alr system activity did not correlate with changes in the accumulation of the native Alr1 protein, indicating that changes in Alr1 protein level do not explain the regulation of Alr system activity. For these reasons, I examined other aspects of Alr1 biology to identify possible mechanisms of Alr1 regulation by Mg supply.

3.7.2 Role of the C-terminal domain in regulation

Forms of Alr1 with lower gel mobility were tentatively identified as phosphorylated in published work (Wachek et al, 2006). Previous workers in this laboratory verified that this change in Alr1 modification was responsive to intracellular Mg supply (Pisat et al, 2009). When Mg-replete cells were transferred to Mg-free medium, the rate at which the Alr1 protein was modified from a lower mobility to a higher mobility form depended on the intracellular Mg supply, as cells that were loaded with Mg prior to transfer showed a slower rate of transition. In addition, the *mnr2* mutation substantially increased the speed of this transition, which is consistent with reduced access to intracellular Mg stores in this strain. Both observations are consistent with two interpretations: first, that the post-translational modification of Alr1 is sensitive to Mg supply, and second, that intracellular Mg (rather than the external concentration) influences this modification.

If the phosphorylation of Alr1 is dependent on Mg supply, it is possible that this process plays a role in regulating the activity of the protein. For this reason, I examined the role of two conserved and phosphorylated serine residues (S847 and S850) in the C-terminal domain of the protein (**Figure 3.7**). By construction of deletions within this

region and complementation assays, I determined that this domain is not essential for basal Alr1 function (**Figure 3.7A**). However, I observed that the deletion of the entire domain moderately decreased the function of the myc-tagged version of the protein **(Figure 3.7B)**. Since changes in function could result from mislocalization of the Alr1 protein, I also examined the subcellular location of YFP-tagged versions of the Alr1 protein (the full length protein and the two deletions) **(Figure 3.10)**. The results indicated that deletion of the C-terminal domain had little effect on the location of the protein, which was still primarily detected at the cell surface in both deficient and replete yeast cells.

Together, my observations suggest that phosphorylation may have a role to play in regulating Alr1 activity, and establish a basic strategy to analyze the contribution of this modification to regulation. It remains to be seen however if the C-terminal domain, and in particular the conserved serine residues within the region, are involved in this process. In the final chapter of this thesis, I will outline experiments designed to determine the role of phosphorylation in Alr1 regulation.

Chapter 4: Discussion

4.1 Model for Mg homeostasis in yeast

The CorA family of Mg channels is widespread in biology, and members play an important role in the regulation of cytosolic Mg concentration. Although the expression of most characterized CorA proteins is constitutive, a previous study (Graschopf et al, 2001) suggested that the expression of the major Mg uptake system Alr1 was regulated by Mg supply. The post-translational control of Alr1 stability was suggested to be the primary mechanism for this regulation. In this work, I investigated some aspects of the regulation of Alr1 accumulation and activity. My findings and those of others in this laboratory suggest a revised model for Mg homeostasis in yeast, which is shown in **Figure 4.1**. In short, this model proposes that Alr1 activity is regulated by cytosolic Mg supply, but that this regulation is probably not a consequence of changes in Alr1 protein accumulation. The evidence for this model is summarized below.

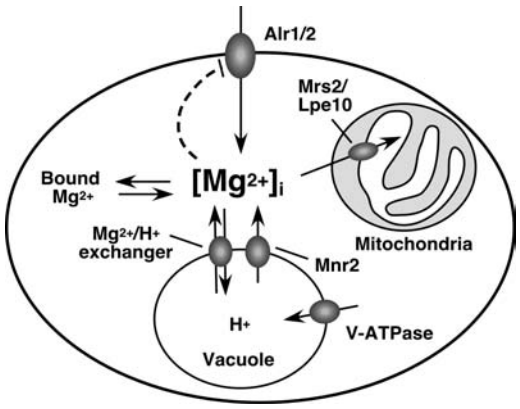

Figure 4.1 Model for Mg homeostasis in yeast. Mg uptake into the cell is mediated by the Alr1 and Alr2 proteins, which contribute Mg to a cytosolic pool of free ionized Mg ($[Mg^{2+}]_i$). This pool is in equilibrium with Mg bound to ligands such as ATP, and can be diverted to other subcellular compartments such as the mitochondria (via the action of the Mrs2/Lpe10 proteins) and the vacuole (via an as-yet unidentified proton-coupled exchanger). The concentration of this cytosolic pool regulates the activity of the Alr1 and Alr2 proteins (dashed line). The Mnr2 protein also regulates the concentration of this pool. In cells lacking Mnr2, the cytosolic Mg pool may decrease, leading to an increase in the activity of the Alr systems. Regulation of Alr activity may be achieved via its post-translational modification, but does not appear to be mediated by altered Alr1 gene expression.

4.2 Effect of Mg supply and Mnr2 on Alr system activity

The Mnr2 protein is a vacuolar transporter which allows the release of Mg from an intracellular storage compartment (most likely the vacuole). In addition to its role in Mg homeostasis, the *mnr2* mutation is also associated with changes in the content of other elements, including that of several divalent cations normally present in yeast

synthetic medium (Pisat et al, 2009). For example, *mnr2* mutants overaccumulated Mn^{2+}, Ca^{2+} and Zn^{2+} when grown in Mg-deficient conditions (Pisat et al, 2009), and the mutant was also more sensitive to these metals. These observations suggested that a low-affinity divalent cation transport system was upregulated in the *mnr2* mutant (Pisat et al, 2009), leading to an increased rate of divalent cation influx. Since the Alr proteins were reported to mediate relatively non-specific divalent cation uptake (MacDiarmid & Gardner, 1998a), it was suspected that the *mnr2* mutation increased the activity of these systems (Pisat et al, 2009).

Recent studies from this laboratory appear to support this model (**Figure 3.6**). Both Mg-deficient yeast cells and *mnr2* mutant strains exhibit increased activity of a cobalt(III)-hexaamine chloride (Cohex) sensitive Ni^{2+} uptake system, indicating the involvement of a CorA-type transport system. Enhanced Cohex-sensitive Ni^{2+} uptake was observed in the *mnr2* mutant, but when this mutation was combined with the *alr1* and *alr2* mutations, the increase in activity was suppressed (**Figure 3.6**). Together, these data indicate that the elevated Cohex-sensitive Ni^{2+} uptake was primarily attributable to the Alr systems, and that the *mnr2* mutation upregulated the activity of this system (rather than a novel independent Ni^{2+} uptake system). These observations are important because they establish that the Alr systems are regulated according to Mg supply, consistent with a role in Mg homeostasis.

4.3 Regulation of Alr1 protein stability

Previous workers in this laboratory had established that *ALR1* mRNA accumulation was not regulated by Mg supply (A. Achari and C. MacDiarmid, unpublished results), and that Mg supply and the *mnr2* mutation had no effect on *ALR1* promoter activity (Pisat, 2009). Both observations are inconsistent with a model in which Alr system activity is increased as a consequence of Mg-responsive gene expression. However, the Alr1-HA protein accumulated to much higher levels in Mg-deficient cells, and its accumulation was enhanced by the *mnr2* mutation (Pisat, 2009), indicating that the stability of the Alr1-HA protein might be affected by Mg supply. If so, this change might contribute to the regulation of Alr system activity.

Consistent with this model, I also observed a large change in Alr1-HA

accumulation in Mg-deficient and replete conditions **(Figure 3.1, 3.2B)**. As previously noted by others in this laboratory (A. Pandey and C. MacDiarmid, unpublished data), the accumulation of Alr1-HA in replete conditions was also substantially increased by the *pep4*, *npi1*, and *doa4* mutations, which presumably prevent the targeting to, and degradation of Alr1-HA in the vacuole lumen. Together, these observations suggest that Alr1-HA was a substrate for ubiquitin-mediated protein degradation. However, I observed a much less pronounced effect of Mg supply and the *doa4* and *pep4* mutations on the accumulation of an N-terminally myc-tagged Alr1 protein (**Figure 3.2A, 3.4**) and the native (untagged) protein **(Figure 3.3C, 3.4)**. A direct comparison of the level of Alr1 protein and Alr1-HA that accumulated in cells expressing both forms indicated that the HA-tagged form was much less abundant than the native form in Mg-replete cells, suggesting that the addition of these tags to the Alr1 protein decreased its accumulation in replete conditions (**Figure 3.4B**). This effect appears to be responsible for the majority of the apparent "regulation" of Alr1-HA accumulation. In addition, while the *mnr2* mutation had a large effect on the accumulation of HA-tagged Alr1 (Pisat, 2009), this mutation had much less effect on the accumulation of the untagged protein (**Figure 3.5**). The *doa4* and *pep4* mutations also had much less effect on the differential accumulation of the untagged Alr1 protein (and the myc-tagged version) than on the HA-tagged version (**Figure 3.4**). All these observations are consistent with a model in which the HA tag destabilized the Alr1 protein in replete conditions, resulting in an artifactually low accumulation of the protein in Mg-replete cells (**Figure 4.2**).

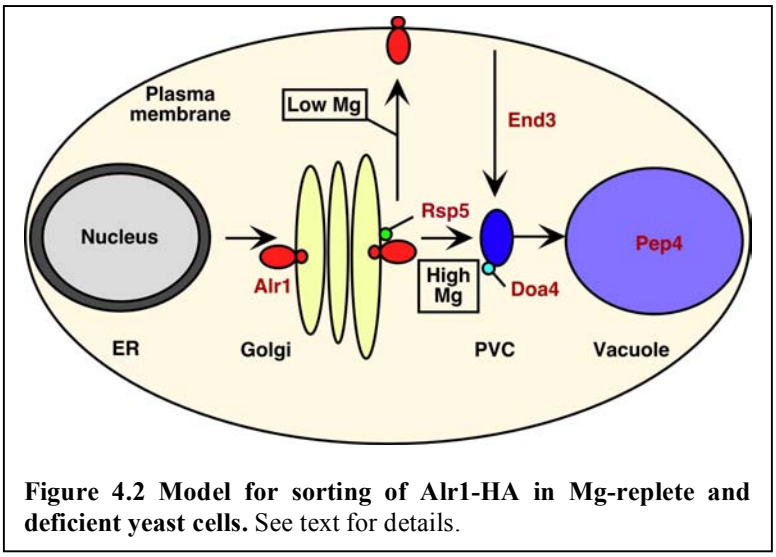

Figure 4.2 Model for sorting of Alr1-HA in Mg-replete and deficient yeast cells. See text for details.

Factors required for the regulated inactivation of some membrane proteins (*e.g.* Rsp5 and Doa4, **Figure 4.2**) are also involved in routine protein quality control

mechanisms within the secretory pathway (Pizzirusso & Chang, 2004). It is possible that the addition of the HA tags simply destabilized the Alr1 protein, causing it to be misfolded. If aberrant forms of Alr1-HA accumulated in the Golgi, these might be recognized by quality-control mechanisms in this compartment, ubiquitinated by Rsp5, and sorted directly to the vacuole for degradation. Another misfolded plasma membrane protein in yeast, Pma1-7, is also sorted to the vacuole via a ubiquitin-dependent pathway (Pizzirusso & Chang, 2004). That the absence of endocytosis had no effect on the stability of Alr1-HA (A. Pandey and C. MacDiarmid, unpublished data) is consistent with the protein being sorted directly from Golgi to vacuole, without transiting the plasma membrane (**Figure 4.2**). While regulated proteins that accumulate in the plasma membrane in permissive conditions (*e.g.,* Gap1) are normally still subject to degradation when conditions change, the stability of the pool of Alr1-HA protein that accumulated in Mg-deficient cells was insensitive to Mg repletion (Pisat, 2009), which further suggests that the processes controlling Alr1-HA accumulation are not primarily designed to regulate the level of Alr1 activity according to Mg supply.

This model raises several questions regarding Alr1-HA behavior. In particular, if the instability of the Alr1-HA protein is an artifact caused by the tag, it is still not clear why severely Mg-deficient cells accumulated more of the Alr1-HA protein than replete cells. There are at least two possible explanations for this phenomenon however. First, it is possible that protein quality control mechanisms become less efficient in severely Mg-deficient cells, leading to the accumulation of proteins that would normally be degraded. Such defects could arise if key steps in these quality control processes were Mg-dependent. In this regard, it is interesting to note that many of the basic processes required for protein trafficking and ubiquitination are ATP-dependent. For example, the covalent addition of ubiquitin to E1 ligase enzymes is ATP-dependent, and may thus depend on access to adequate Mg as a cofactor for this reaction (Hicke, 1997). Second, it is possible that the conformation of the Alr1-HA protein itself is Mg-dependent, which could directly affect its stability. Mg has been reported to cause major changes in the conformation of the related CorA protein from *Thermatoga maritima*, and these changes are believed to play a role in the regulation of activity (Payandeh et al, 2008; Payandeh & Pai, 2006b). The Mg-binding residues required to mediate these conformation changes

are tightly conserved in the Alr1 protein (C. MacDiarmid, personal communication). If Mg availability has the same effect on Alr1-HA conformation as it does for CorA, it is possible that in replete conditions, this protein takes on a conformation that is less intrinsically stable, or that exposes hydrophobic residues that are targets for protein quality control mechanisms. In this way, the stability of the protein could become abnormally responsive to Mg supply.

Another aspect of this work which raises several questions is the observation that the co-expression of Alr1-HA with the untagged Alr1 protein altered Alr1-HA accumulation (**Figure 3.2B**). As initially observed by A. Pandey (**Figure 1.1**), when Alr1-HA was expressed in a wild-type strain, the protein exhibited differential accumulation in response to Mg supply. However, when Alr1-HA was expressed in an *alr1 alr2* mutant, the protein accumulated to a much higher level in both replete and deficient cells, and no consistent effect of Mg was observed. The *alr1* and *alr2* mutations also affected the accumulation of the myc-tagged Alr1 protein (**Figure 3.2A**), although the specific nature and the severity of this effect was different (myc-Alr1 expressed in an *alr1 alr2* mutant accumulated to a lower level in deficient vs replete conditions, while Alr1-HA accumulated to a much higher level in replete cells of the same mutant strain).

One possible interpretation of these findings might be that in replete cells, the wild-type version of Alr1 or Alr2 can confer instability on the Alr1-HA protein by restoring a regulatory process that was prevented by the addition of the tag. According to this model, complexes made up entirely of the Alr1-HA subunit would be more stable because their downregulation would be prevented (for example, by the inhibition of ubiquitination). The regulatory function might however be supplied *in trans* by the association of a normal subunit with a modified subunit within a heteromeric complex. For example, the addition of a single ubiquitin tag to one unmodified subunit might be sufficient to allow sorting of the entire complex to the vacuole for degradation.

Although this model was initially attractive, the finding that the N-terminally tagged and unmodified Alr1 proteins were not subject to strong regulation of their accumulation challenges the validity of the idea that the HA tags inhibit a normal regulatory process. Given this finding, it is somewhat difficult to understand why the co-expression of native and HA-tagged Alr1 decreased the accumulation of the tagged

protein (**Figure 3.2B**). One possibility is that the HA-tagged version does not easily assemble into heteromeric complexes with the native protein (perhaps due to small differences in the conformation of the tagged protein, or steric hindrance of assembly by the HA tags). If this was the case, then complexes of native Alr1 might predominantly form, from which the tagged protein would be excluded. This effect might decrease the stability of the modified protein, as unassembled subunits of Alr1-HA might be preferentially degraded by protein quality control mechanisms, or might fail to associate with lipid rafts, a process that is required for sorting of some proteins to the plasma membrane (Bagnat et al, 2001). However, this explanation raises the question of why Alr1-HA subunits would not form stable homomeric complexes under these conditions, as they apparently do in *alr1 alr2* mutant cells expressing only Alr1-HA. Thus, at present there is no clear explanation for the behavior of the HA-tagged protein. One clear implication of these results however, is that experiments utilizing epitope-tagged proteins must be carefully controlled to ensure the reliability of the results observed.

4.4 Regulation of Alr1 activity by Mg supply

My experiments demonstrate that native Alr1 accumulation is little affected by Mg supply **(Figure 3.5)**. In addition, the effect of Mg supply and the *mnr2* mutation on Alr system activity did not correlate well with changes in the accumulation of the native Alr1 protein, indicating that changes in Alr1 protein accumulation do not explain the regulation of activity **(Figure 3.5, 3.6)**. Specifically, *mnr2* mutant cells supplied with 100 μM Mg showed a substantial increase in Alr system activity over the wild-type (**Figure 3.6C**), but under the same conditions, there was no corresponding increase in the accumulation of the native Alr1 protein in the mutant strain (**Figure 3.5**). To better understand the mechanism of this observed regulation, I performed some experiments to examine the role of other aspects of Alr1 biology.

4.4.1 Analysis of the role of phosphorylation in Alr1 regulation

I and others noticed changes in the gel mobility of the Alr1 protein in cells supplied with different Mg concentrations. Alr1 with lower gel mobility were previously suggested to be phosphorylated based on an increased gel mobility when these forms

were treated with lambda phosphatase (Graschopf et al, 2001; Wachek et al, 2006). Previous workers in this laboratory determined that this change in Alr1 modification was responsive to intracellular Mg supply (Pisat, 2009). When Mg -replete cells were transferred to Mg -free medium, the rate at which the Alr1 protein was modified from a lower mobility to a higher mobility form (indicating phosphorylation) depended on the intracellular Mg supply, as cells that were loaded with Mg prior to transfer showed a slower rate of transition. In addition, the *mnr2* mutation substantially increased the speed of this transition, which is consistent with reduced access to intracellular Mg stores in this strain. Both observations are consistent with two interpretations: first, that the post-translational modification of Alr1 is sensitive to Mg supply, and second, that intracellular Mg (rather than the external Mg concentration) influences this modification.

4.4.1.1 Construction of C-terminal domain deletions

If the phosphorylation of Alr1 is dependent on Mg supply, it is possible that this process plays a role in regulating the activity of the protein. As a first step in this analysis, I examined the role of the C-terminal domain of the Alr1 protein, which contains two conserved serine residues (S847 and S850) that are reportedly phosphorylated (**Figure 3.7**). I constructed two deletions, one which removed most of the predicted cytosolic domain (Δ806), and another that removed a smaller region containing both conserved serine residues (Δ828). The C-terminal domain was not essential for basic Alr1 function (**Figure 3.8A**), since both constructs at least partially complemented the growth defect of an *alr1 alr2* mutant. As shown in **Figure 3.8B**, deletion of almost the entire C-terminal domain (Δ806) had a small effect on growth in liquid medium with a reduced Mg concentration, and was associated with a substantial decrease in the accumulation of this protein (**Figure 3.9A**). However, the smaller deletion (Δ828) had no effect on Alr1 function.

To verify that the deletions did not prevent the Alr1 protein reaching the cell surface, three YFP-tagged versions of Alr1 were also constructed. All three proteins were detected at the plasma membrane in both deficient and replete cells. However, somewhat more protein of the deleted versions was observed to accumulate within an intracellular compartment (**Figure 3.10**), suggesting that somewhat more of the deleted proteins may

reach this compartment. This result is consistent with the small decrease in protein accumulation that was associated with the deletions in the YFP-tagged proteins, particularly for YFP-Alr1Δ806 (**Figure 3.9B**). Despite these minor phenotypic differences, my results indicate that the Alr1-Δ828 construct in particular provides a good starting point for analysis of the role of the C-terminal domain in Alr1 regulation.

4.4.2 Strategy to determine the role of S847 and S850 in Alr1 regulation

The two conserved serine residues in the C terminal domain of Alr1 are potential targets for phosphorylation (Ser^{847} and Ser^{850}, **Figure 3.7**). My data shows other residues in Alr1 may also be subject to Mg-dependent phosphorylation, as deletion of both these residues did not prevent the shift in protein mobility associated with changes in Mg supply (**Figure 3.9**). Thus, these two residues are not the only candidates for involvement in regulation. To determine their role, I would mutate both to alanine using PCR-mediated site-directed mutagenesis. This change would have the effect of preventing phosphorylation, while making a minimal change to the physical properties (size and charge) of the residues at these positions. The effect of these mutations on Alr1 activity would then be determined using Ni^{2+} uptake assays. To ensure that subtle effects of the mutations on regulation were not missed, *alr1* mutant strains expressing either the single or the double mutant would be grown over a range of Mg concentrations before measurement of Alr activity. As a control, I would also perform a Western blot experiment using the anti-Alr1 antibody to verify that the mutations did not affect the accumulation of Alr1. In addition to the serine mutants, I would also examine myc-Alr1

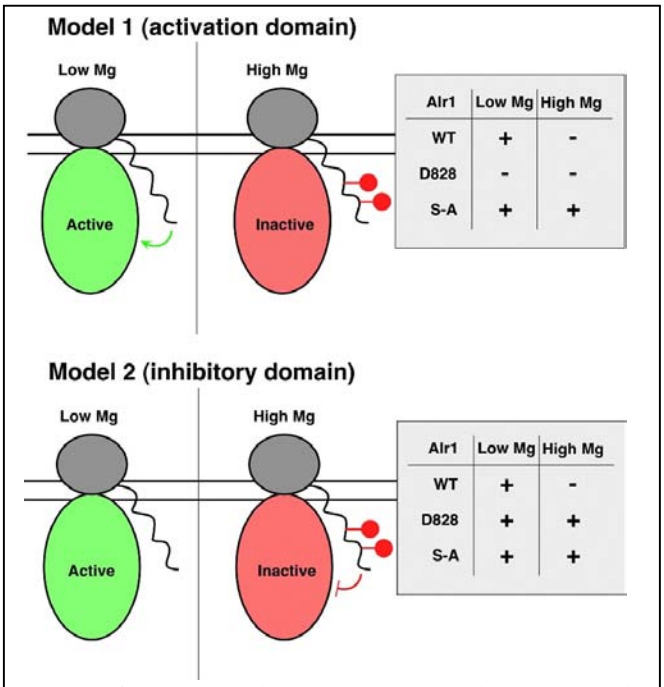

Figure 4.3 Models for the function of the Alr1 C-terminal domain. See text for details.

Δ806, Δ828, and full-length versions in this experiment. As discussed below, the behavior of the modified versions of Alr1 should reveal if the domain is important for regulation, as well as the mode of action of this domain (repression or activation of activity).

I would predict at least two possible outcomes for this experiment (**Figure 4.3**). First, it is possible that the Alr1 C-terminal domain *activates* Alr1 under conditions of Mg-deficiency (**Figure 4.3,** model 1). According to this model, eliminating the C-terminal activating domain entirely (by its deletion) would be predicted to reduce Alr1 activity under Mg-deficient conditions. In addition, if the two conserved serines within this domain are phosphorylated in replete conditions, this process would be expected to inactivate the C-terminal domain, preventing it from activating Alr1 under deficient conditions. If this model is correct, mutating the serines to prevent their phosphorylation would permanently activate the C-terminal domain, and thus up-regulate Alr1 under Mg-replete conditions.

Alternatively, it is possible that the C-terminal domain is responsible for *inhibiting* Alr1 activity under Mg-replete conditions (**Figure 4.3,** model 2**)**. If this is the case, deleting this domain will activate Alr1 under Mg-replete conditions. In addition, this model would predict that under replete conditions, phosphorylation of the serines in this domain is required for its inhibitory function. If this is the case, mutations that prevent phosphorylation will prevent this inhibitory function, and Alr1 will be activated in Mg-replete conditions. Thus, mutation of the serine residues would not discriminate between inhibitory or activatory roles for the C-terminal domain, but when compared with the results of deletion analysis, the experiment should allow me to assign a function to this domain.

4.4.3 Identification of additional phosphorylated residues in Alr1

The two conserved serine residues that I have focused on in this study are not the only residues in Alr1 implicated as targets for phosphorylation. Studies of the yeast proteome using mass spectrometry identified several potentially phosphorylated residues in Alr1 (Bodenmiller et al, 2008). In addition to Ser^{847} and Ser^{850}, the Phosphopep database also identifies a strong signal at Ser^{176} and Ser^{185}, as well as several more

ambiguous signals at other serine and threonine residues within the N-terminal cytosolic domain. Since peptides corresponding to less than 10% of the Alr1 sequence were identified in this database, there are potentially many more modified residues in the Alr1 protein. The technical limitations of large-scale proteomic studies are one reason for the relatively low amount of available data on Alr1 (proteins expressed at relatively low levels are less likely to be represented in these datasets). For this reason, further investigations of Alr1 modification using mass spectrometry would need to focus on improving the recovery of Alr1 peptides. To this end, a good strategy would be to purify the Alr1 protein from Mg-deficient and Mg-replete yeast cells before analysis with mass spectrometry. This might be achieved by the addition of domains to simplify purification of the protein, for example the TAP tag (tandem affinity purification) (Puig et al, 2001). This system relies on a two-step purification procedure to increase protein purity. The phosphorylation of Alr1 in replete conditions could then be verified more definitively by the use of western blotting with phospho-serine or phospho-threonine antibodies. I would predict that based on earlier results, these antibodies should cross-react with the lower mobility form of Alr1, but not with the higher mobility form. The purified proteins would then be digested with trypsin prior to mass spectrometry. Peptides showing a difference in phosphorylation in Mg-replete cells vs deficient cells would be easily identified. Phosphorylated residues could then be subject to a similar strategy of genetic analysis as described previously to determine their role (if any) in regulation.

4.4.4 Identification of kinase enzymes required for Alr1 phosphorylation

If Alr1 activity is regulated by phosphorylation, this finding would suggest that yeast cells can respond to Mg deficiency by the activation of specific signal transduction pathways, which in turn lead to the activation of the specific kinases responsible for Alr1 phosphorylation. There is little information available to suggest what pathways might be responsible for this process, but their identification would be an important goal for future research, because it may allow us to identify the mechanism by which yeast sense Mg-deficient conditions. This in turn may allow us to better understand these process in higher eukaryotes, where gene regulation by Mg supply is important for Mg homeostasis (Zhou & Clapham, 2009).

As a first step in this investigation, I would examine the Alr1 sequence for consensus protein kinase recognition sites, particularly in areas that contain residues likely to be phosphorylated (identified as described above). Kinase recognition sites can be located using motifs based on the sequences of known sites using programs such as NetPhosYeast (Ingrell et al, 2007). In the Alr1 sequence, NetPhosYeast identified the two conserved serine residues in the Alr1 C-terminal domain as potentially phosphorylated, as well as many more potential sites within the protein (data not shown). However, currently this program does not report which kinase enzymes would be expected to phosphorylate the sites that it identifies. To identify these kinases, I would use a genetic strategy that takes advantage of many resources available for yeast researchers. A set of viable mutants lacking single kinases (obtained from commercial sources) would be used to screen for those affecting Alr1 regulation. The mutants and wild-type control strain would be grown in Mg-deficient and replete medium, and Alr1 activity would be measured by Ni^{2+} uptake analysis. These assays would allow me to rapidly identify any mutants with reduced ability to either induce Alr1 activity in deficient conditions, or repress it in replete conditions. I would then directly assay these mutants to determine their effect on Alr1 phosphorylation. Using TAP-tagged or other modified versions of Alr1, I would purify the protein as described previously, and determine if the absence of the kinase prevented Alr1 phosphoryation at particular residues. Initially, these studies would be performed using immunoblotting with anti-phosphoserine or threonine antibodies to detect changes in phosphorylation. However, the potential complexity of Alr1 modification means that I may need to use mass spectrometry to detect the effect of the mutations on specific residues.

Once a kinase responsible for Alr1 phosphorylation is identified, I will verify its involvement by studying the effects of other mutations which are known to affect the signal transduction pathway in which the kinase participates. In this regard, a study in yeast has revealed an interesting pathway which may be involved in Mg homeostasis (Wiesenberger et al, 2007). A genome-wide screen for genes induced by Mg-deficiency identified a large number of upregulated genes, many of which were also induced by high Ca^{2+} concentrations. It was shown that the induction of these genes in Mg-starved cells was dependent on external Ca^{2+}, and that Mg-starvation triggered rapid Ca^{2+} influx,

which induced the activity of the Crz1p/calcineurin signal transduction pathway. Although this response could simply reflect the ability of Mg to block Ca^{2+} influx via the Alr proteins (Cui et al, 2009), it could also indicate that yeast cells can respond to Mg via the activity of a preexisting signal transduction pathway. Yeast cells sense high Ca concentrations within the cytosol via the activity of calmodulin (Cmd1), which binds calcium ions and activates the calcineurin protein phosphatase. This enzyme in turn dephosphorylates the transcriptional activator Crz1, which upregulates transcription of a set of calcium responsive genes (Matheos et al, 1997). It is possible that some of these genes, rather than being required for Ca^{2+} homeostasis, are actually involved in Mg homeostasis. It is also possible that the calcineurin complex is responsible for directly dephosphoryating and thereby activating Alr1. An influx of Ca^{2+} and the activation of the Ca^{2+}/calmodulin pathway could thus act as a convenient signal for an external Mg deficiency. This model could be tested by examining the ability of calmodulin pathway mutants to activate Alr1 in Mg-deficient conditions, as well as determining their effect on Alr1 phosphorylation.

4.5 Alternative mechanisms of Alr1 regulation

If the strategy described above was unsuccessful at identifying a link between phosphorylation and Alr1 regulation, it is possible that (like the *T. maritima* CorA protein) the Alr1 protein is primarily regulated via the direct binding of Mg to its cytosolic N-terminal domain. Structural analysis of this protein revealed two apparent Mg-binding sites at each subunit interface of the CorA complex (M1 and M2) (Payandeh & Pai, 2006a). Mg ions were proposed to bind in the M1 site via direct co-ordination with the Asp89 and Asp253 residues, while the M2 site bound Mg via indirect contacts through Asp175 and Asp179. Additional residues also contributed to ion binding in each site. In the M2 domain for example, Glu88 and His257 made contacts with Mg via water molecules. The role of these sites in regulating the activity of the CorA protein is supported by genetic analysis. For example, when Asp 253 in M1 was replaced with a hydrophobic phenylalanine residue, which was predicted to prevent the M1 domain binding Mg, the protein became insensitive to inhibition by Mg ions (as measured by *in vitro* transport assays), and its ability to complement a *S. typimurium corA* mutation was

increased. Conversely, when Asp253 was replaced with a positively charged lysine residue (to simulate the presence of a Mg ion), the protein was essentially inactive in both of the above assays.

Sequence alignment of the C-terminal domain of the Alr1 and Mnr2 proteins with *T. maritima* CorA demonstrate that these proteins are significantly conserved. In particular, those residues implicated in forming the M1 and M2 Mg-binding sites in CorA are conserved in both the yeast proteins. To determine the role of these sites in Alr1 regulation, I would perform a similar genetic analysis of the Alr1 protein, replacing the conserved residues with alternatives predicted to produce changes in the regulation of the protein. For example, the equivalent residue to Asp253 of CorA is Asp694 in Alr1. I would construct mutant versions of Alr1 in which this residue is converted to phenylalanine or lysine, which would be expected to activate and inactivate the protein respectively. The effect on activity would be measured using Ni uptake assays with *alr1* mutant strains expressing the mutant proteins and grown in Mg-replete or deficient conditions. These experiments would reveal the extent to which these sensor domains contribute to the regulation that we observed.

In addition to their effect on Alr1 activity, these genetic changes would be expected to have effects on the conformation of the protein in Mg-containing and Mg-free buffers. Successful purification of Alr1 (as described above) would enable me to perform biochemical assays to investigate the effect of Mg ions on the conformation of the Alr1 protein. The CorA protein has been reported to undergo structural changes in response to the presence of Mg, which make the protein more resistant to protease cleavage. To determine if the Alr1 protein also undergoes Mg-dependent conformational changes, the purified protein could be incubated in buffer containing or lacking Mg, then incubated with a selection of proteases. The digestion products would be separated using SDS-PAGE and visualized by silver staining to determine the effect of Mg on protease sensitivity. Once an appropriate combination of protease and conditions is found to reveal these changes, the specificity of the effect would be determined by including the Mg-binding site mutants in the assay. Mutations which prevent Mg binding to the protein might increase protease sensitivity (since they would be predicted to generate a more open conformation), while mutations that simulate constitutively bound Mg should

increase protease resistance. In this way, I could verify that the mutations were having the expected effects on Alr1 structure and function.

BIBLIOGRAPHY

Amerik AY, Nowak J, Swaminathan S, Hochstrasser M (2000) The Doa4 deubiquitinating enzyme is functionally linked to the vacuolar protein-sorting and endocytic pathways. *Mol Biol Cell* **11**(10)**:** 3365-3380

Bagnat M, Chang A, Simons K (2001) Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. *Mol Biol Cell* **12**(12)**:** 4129-4138

Bodenmiller B, Campbell D, Gerrits B, Lam H, Jovanovic M, Picotti P, Schlapbach R, Aebersold R (2008) PhosphoPep-a database of protein phosphorylation sites in model organisms. *Nat Biotechnol* **26**(12)**:** 1339-1340

Bodenmiller B, Malmstrom J, Gerrits B, Campbell D, Lam H, Schmidt A, Rinner O, Mueller LN, Shannon PT, Pedrioli PG, Panse C, Lee HK, Schlapbach R, Aebersold R (2007) PhosphoPep-a phosphoproteome resource for systems biology research in Drosophila Kc167 cells. *Mol Syst Biol* **3:** 139

Bonneaud N, Ozier-Kalogeropoulos O, Li GY, Labouesse M, Minvielle-Sebastia L, Lacroute F (1991) A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae/E. coli* shuttle vectors. *Yeast* **7**(6)**:** 609-615

Bui DM, Gregan J, Jarosch E, Ragnini A, Schweyen RJ (1999) The bacterial magnesium transporter CorA can functionally substitute for its putative homologue Mrs2p in the yeast inner mitochondrial membrane. *J Biol Chem* **274**(29)**:** 20438-20443.

Carafoli E, Brini M (2000) Calcium pumps: structural basis for and mechanism of calcium transmembrane transport. *Curr Opin Chem Biol* **4**(2)**:** 152-161

Chubanov V, Waldegger S, Mederos y Schnitzler M, Vitzthum H, Sassen MC, Seyberth HW, Konrad M, Gudermann T (2004) Disruption of TRPM6/TRPM7 complex formation by a mutation in the TRPM6 gene causes hypomagnesemia with secondary hypocalcemia. *Proc Natl Acad Sci USA* **101**(9)**:** 2894-2899

Cowan JA (2002) Structural and catalytic chemistry of magnesium-dependent enzymes. *Biometals* **15**(3)**:** 225-235

Cromie MJ, Shi Y, Latifi T, Groisman EA (2006) An RNA sensor for intracellular Mg^{2+}. *Cell* **125**(1)**:** 71-84

Cui J, Kaandorp JA, Ositelu OO, Beaudry V, Knight A, Nanfack YF, Cunningham KW (2009) Simulating calcium influx and free calcium concentrations in yeast. *Cell Calcium* **45**(2)**:** 123-132

De Craene JO, Soetens O, Andre B (2001) The Npr1 kinase controls biosynthetic and

endocytic sorting of the yeast Gap1 permease. *J Biol Chem* **276**(47)**:** 43939-43948

Delhaize E, Ryan PR (1995) Aluminium toxicity in plants. *Plant Physiol* **107:** 115-121

Deng W, Luo K, Li D, Zheng X, Wei X, Smith W, Thammina C, Lu L, Li Y, Pei Y (2006) Overexpression of an Arabidopsis magnesium transport gene, AtMGT1, in *Nicotiana benthamiana* confers Al tolerance. *J Exp Bot* **57**(15)**:** 4235-4243

Drummond RSM, Tutone A, Li YC, Gardner RC (2006) A putative magnesium transporter AtMRS2-11 is localized to the plant chloroplast envelope membrane system. *Plant Science* **170**(1)**:** 78-89

Dunn R, Hicke L (2001) Multiple roles for Rsp5p-dependent ubiquitination at the internalization step of endocytosis. *J Biol Chem* **276**(28)**:** 25974-25981

Dupre S, Haguenauer-Tsapis R (2001) Deubiquitination step in the endocytic pathway of yeast plasma membrane proteins: crucial role of Doa4p ubiquitin isopeptidase. *Mol Cell Biol* **21**(14)**:** 4482-4494

Eshaghi S, Niegowski D, Kohl A, Martinez Molina D, Lesley SA, Nordlund P (2006) Crystal structure of a divalent metal ion transporter CorA at 2.9 angstrom resolution. *Science* **313**(5785)**:** 354-357

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nat Biotechnol* **20**(3)**:** 301-305

Fischer ES, Bremer E (1993) Influence of Magnesium-Deficiency on Rates of Leaf Expansion, Starch and Sucrose Accumulation, and Net Assimilation in *Phaseolus vulgaris*. *Physiologia Plantarum* **89**(2)**:** 271-276

Flowers TJ (2004) Improving crop salt tolerance. *J Exp Bot* **55**(396)**:** 307-319

Franklin AJ (2007) Characterization of Putative Magnesium Transport systems in yeast. MS Thesis, Biology, University of Missouri-St Louis, St Louis.

Froschauer EM, Kolisek M, Dieterich F, Schweigel M, Schweyen RJ (2004) Fluorescence measurements of free [Mg^{2+}] by use of MagFura-2 in *Salmonella enterica*. *FEMS Microbiol Lett* **237**(1)**:** 49-55

Galan JM, Haguenauer-Tsapis R (1997) Ubiquitin Lys63 is involved in ubiquitination of a yeast plasma membrane protein. *Embo J* **16**(19)**:** 5847-5854

Garcia Vescovi E, Soncini FC, Groisman EA (1996) Mg^{2+} as an extracellular signal: environmental regulation of *Salmonella* virulence. *Cell* **84**(1)**:** 165-174.

Gibson MM, Bagga DA, Miller CG, Maguire ME (1991) Magnesium transport in *Salmonella typhimurium*: the influence of new mutations conferring Co^{2+} resistance on the CorA Mg^{2+} transport system. *Mol Microbiol* **5**(11)**:** 2753-2762

Gietz RD, St. Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucl Acids Res* **8:** 1425

Gitan RS, Eide DJ (2000) Zinc-regulated ubiquitin conjugation signals endocytosis of the yeast *ZRT1* zinc transporter. *Biochem J* **346 Pt 2:** 329-336

Goytain A, Quamme GA (2005a) Functional characterization of human SLC41A1, a Mg^{2+} transporter with similarity to prokaryotic MgtE Mg^{2+} transporters. *Physiol Genomics* **21**(3)**:** 337-342

Goytain A, Quamme GA (2005b) Functional characterization of the human solute carrier, SLC41A2. *Biochem Biophys Res Commun* **330**(3)**:** 701-705

Goytain A, Quamme GA (2005c) Identification and characterization of a novel mammalian Mg^{2+} transporter with channel-like properties. *BMC Genomics* **6**(1)**:** 48

Graschopf A, Stadler JA, Hoellerer MK, Eder S, Sieghardt M, Kohlwein SD, Schweyen RJ (2001) The yeast plasma membrane protein Alr1 controls Mg^{2+} homeostasis and is subject to Mg^{2+}-dependent control of its synthesis and degradation. *J Biol Chem* **276**(19)**:** 16216-16222

Gregan J, Bui DM, Pillich R, Fink M, Zsurka G, Schweyen RJ (2001) The mitochondrial inner membrane protein Lpe10p, a homologue of Mrs2p, is essential for magnesium homeostasis and group II intron splicing in yeast. *Mol Gen Genet* **264**(6)**:** 773-781.

Grenson M (1983) Study of the positive control of the general amino-acid permease and other ammonia-sensitive uptake systems by the product of the *NPR1* gene in the yeast *Saccharomyces cerevisiae*. *Eur J Biochem* **133**(1)**:** 141-144

Grenson M (1992) Amino acid transporters in yeast: structure, function and regulation. In *Molecular aspects of transport proteins*, Pont JJHHMD (ed), pp 219-245. Amsterdam: Elsevier Science Publishers

Grimme H (1983) Aluminum Induced Magnesium-Deficiency in Oats. *Zeitschrift Fur Pflanzenernahrung Und Bodenkunde* **146**(5)**:** 666-676

Grinstein S, Wieczorek H (1994) Cation antiports of animal plasma membranes. *J Exp Biol* **196:** 307-318

Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. *J Bacteriol* **183**(6)**:** 1835-1842

Groisman EA, Chiao E, Lipps CJ, Heffron F (1989) *Salmonella typhimurium* phoP virulence gene is a transcriptional regulator. *Proc Natl Acad Sci U S A* **86**(18)**:** 7077-7081

Hartwig A (2001) Role of magnesium in genomic stability. *Mutat Res* **475**(1-2)**:** 113-121

Haynes WJ, Kung C, Saimi Y, Preston RR (2002) An exchanger-like protein underlies the large Mg^{2+} current in *Paramecium*. *Proc Natl Acad Sci USA* **99**(24)**:** 15717-15722

Hein C, Springael JY, Volland C, Haguenauer-Tsapis R, Andre B (1995) *NPl1*, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. *Mol Microbiol* **18**(1)**:** 77-87

Hermans C, Johnson GN, Strasser RJ, Verbruggen N (2004) Physiological characterisation of magnesium deficiency in sugar beet: acclimation to low magnesium differentially affects photosystems I and II. *Planta* **220**(2)**:** 344-355

Hermosura MC, Nayakanti H, Dorovkov MV, Calderon FR, Ryazanov AG, Haymer DS, Garruto RM (2005) A TRPM7 variant shows altered sensitivity to magnesium that may contribute to the pathogenesis of two Guamanian neurodegenerative disorders. *Proc Natl Acad Sci U S A* **102**(32)**:** 11510-11515

Hicke L (1997) Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. *Faseb J* **11**(14)**:** 1215-1226

Hmiel SP, Snavely MD, Florer JB, Maguire ME, Miller CG (1989) Magnesium transport in *Salmonella typhimurium*: genetic characterization and cloning of three magnesium transport loci. *J Bacteriol* **171**(9)**:** 4742-4751

Hmiel SP, Snavely MD, Miller CG, Maguire ME (1986) Magnesium transport in *Salmonella typhimurium*: characterization of magnesium influx and cloning of a transport gene. *J Bacteriol* **168**(3)**:** 1444-1450

Hoenderop JG, Bindels RJ (2005) Epithelial Ca^{2+} and Mg^{2+} channels in health and disease. *J Am Soc Nephrol* **16**(1)**:** 15-26

Ingrell CR, Miller ML, Jensen ON, Blom N (2007) NetPhosYeast: prediction of protein phosphorylation sites in yeast. *Bioinformatics* **23**(7)**:** 895-897

Jauniaux JC, Grenson M (1990) *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. *Eur J Biochem* **190**(1)**:** 39-44

Jones, E.W., Zubenko, G.S. and Parker, R.R. (1982) *PEP4* gene function is required for expression of several vacuolar hydrolases in *Saccharomyces cerevisiae*. *Genetics*, **102**, 665-677.

Kehres DG, Lawyer CH, Maguire ME (1998) The CorA magnesium transporter gene family. *Microb Comp Genomics* **3**(3)**:** 151-169

Kehres DG, Maguire ME (2002) Structure, properties and regulation of magnesium transport proteins. *Biometals* **15**(3)**:** 261-270

Keltjens WG, Tan K (1993) Interaction between aluminium, magnesium and calcium with different monocotyledonous and dicotyledon plant species. *Plant and Soil* **155/156:** 485-488

King NL, Deutsch EW, Ranish JA, Nesvizhskii AI, Eddes JS, Mallick P, Eng J, Desiere F, Flory M, Martin DB, Kim B, Lee H, Raught B, Aebersold R (2006) Analysis of the *Saccharomyces cerevisiae* proteome with PeptideAtlas. *Genome Biol* **7**(11)**:** R106

Knoop V, Groth-Malonek M, Gebert M, Eifler K, Weyand K (2005) Transport of magnesium and other divalent cations: evolution of the 2-TM-GxN proteins in the MIT superfamily. *Mol Genet Genomics* **274**(3)**:** 205-216

Kolisek M, Launay P, Beck A, Sponder G, Serafini N, Brenkus M, Froschauer EM, Martens H, Fleig A, Schweigel M (2008) SLC41A1 is a novel mammalian Mg^{2+} carrier. *J Biol Chem* **283**(23)**:** 16235-16247

Kolisek M, Zsurka G, Samaj J, Weghuber J, Schweyen RJ, Schweigel M (2003) Mrs2p is an essential component of the major electrophoretic Mg^{2+} influx system in mitochondria. *EMBO J* **22**(6)**:** 1235-1244

Konrad M, Schlingmann KP, Gudermann T (2004) Insights into the molecular nature of magnesium homeostasis. *Am J Physiol Renal Physiol* **286**(4)**:** F599-605

Laing W, Greer D, Sun O, Beets P, Lowe A, Payn T (2000) Physiological impacts of Mg deficiency in *Pinus radiata*: growth and photosynthesis. *New Phytologist* **146**(1)**:** 47-57

Laires MJ, Monteiro CP, Bicho M (2004) Role of cellular magnesium in health and human disease. *Front Biosci* **9:** 262-276

Li L, Tutone AF, Drummond RS, Gardner RC, Luan S (2001a) A novel family of magnesium transport genes in *Arabidopsis*. *Plant Cell* **13**(12)**:** 2761-2775.

Li LG, Sokolov LN, Yang YH, Li DP, Ting J, Pandy GK, Luan S (2008) A mitochondrial magnesium transporter functions in Arabidopsis pollen development. *Molecular Plant* **1**(4)**:** 675-685

Li LG, Tutone AF, Drummond RSM, Gardner RC, Luan S (2001b) A novel family of magnesium transport genes in Arabidopsis. *Plant Cell* **13**(12)**:** 2761-2775

Lunin VV, Dobrovetsky E, Khutoreskaya G, Zhang R, Joachimiak A, Doyle DA, Bochkarev A, Maguire ME, Edwards AM, Koth CM (2006) Crystal structure of the CorA Mg^{2+} transporter. *Nature* **440**(7085)**:** 833-837

MacDiarmid CW (1997) Aluminium toxicity and resistance in *Saccharomyces cerevisiae.* PhD dissertation, Cellular and Molecular Biology, University of Auckland, New Zealand.

MacDiarmid CW, Gardner RC (1996) Al toxicity in yeast. A role for Mg? *Plant Physiol* **112**(3)**:** 1101-1109

MacDiarmid CW, Gardner RC (1998a) Overexpression of the *Saccharomyces cerevisiae* magnesium transport system confers resistance to aluminum ion. *J Biol Chem* **273**(3)**:** 1727-1732

MacDiarmid CW, Gardner RC (1998b) Overexpression of the *Saccharomyces cerevisiae* magnesium transport system confers resistance to aluminum ion. *J Biol Chem* **273**(3)**:** 1727-1732

Maguire ME (2006) Magnesium transporters: properties, regulation and structure. *Front Biosci* **11:** 3149-3163

Maguire ME, Cowan JA (2002) Magnesium chemistry and biochemistry. *Biometals* **15**(3)**:** 203-210

Mao DD, Tian LF, Li LG, Chen J, Deng PY, Li DP, Luan S (2008) AtMGT7: An Arabidopsis Gene Encoding a Low-Affinity Magnesium Transporter. *J Integr Plant Biol* **50**(12)**:** 1530-1538

Marschner H (1995) *Mineral Nutrition of Higher Plants.*, 2nd edn. Boston: Academic Press.

Marschner H, Cakmak I (1989) High Light-Intensity Enhances Chlorosis and Necrosis in Leaves of Zinc, Potassium, and Magnesium Deficient Bean (Phaseolus-Vulgaris) Plants. *Journal of Plant Physiology* **134**(3)**:** 308-315

Maser P, Thomine S, Schroeder JI, Ward JM, Hirschi K, Sze H, Talke IN, Amtmann A, Maathuis FJ, Sanders D, Harper JF, Tchieu J, Gribskov M, Persans MW, Salt DE, Kim SA, Guerinot ML (2001) Phylogenetic relationships within cation transporter families of *Arabidopsis*. *Plant Physiol* **126**(4)**:** 1646-1667.

Matheos DP, Kingsbury TJ, Ahsan US, Cunningham KW (1997) Tcn1p/Crz1p, a calcineurin-dependent transcription factor that differentially regulates gene expression in *Saccharomyces cerevisiae*. *Genes Dev* **11**(24)**:** 3445-3458

Matsumoto H (2000) Cell biology of aluminum toxicity and tolerance in higher plants. *Int Rev Cytol* **200:** 1-46

Mengel K KE (2001) *Principles of plant nutrition*, 5 edn. Dordrecht: Kluwer Academic Publishers.

Miller SI (1991) PhoP/PhoQ: macrophage-specific modulators of Salmonella virulence? *Mol Microbiol* **5**(9)**:** 2073-2078

Moncrief MB, Maguire ME (1998) Magnesium and the role of MgtC in growth of *Salmonella typhimurium*. *Infect Immun* **66**(8)**:** 3802-3809

Monteilh-Zoller MK, Hermosura MC, Nadler MJ, Scharenberg AM, Penner R, Fleig A (2003) TRPM7 provides an ion channel mechanism for cellular entry of trace metal ions. *J Gen Physiol* **121**(1)**:** 49-60

Montell C (2003) Mg^{2+} homeostasis: the Mg^{2+}-nificent TRPM chanzymes. *Curr Biol* **13**(20)**:** R799-801

Morvan J, Froissard M, Haguenauer-Tsapis R, Urban-Grimal D (2004) The ubiquitin ligase Rsp5p is required for modification and sorting of membrane proteins into multivesicular bodies. *Traffic* **5**(5)**:** 383-392

Nadler MJ, Hermosura MC, Inabe K, Perraud AL, Zhu Q, Stokes AJ, Kurosaki T, Kinet JP, Penner R, Scharenberg AM, Fleig A (2001) LTRPC7 is a Mg-ATP-regulated divalent cation channel required for cell viability. *Nature* **411**(6837)**:** 590-595

Niegowski D, Eshaghi S (2007) The CorA family: structure and function revisited. *Cell Mol Life Sci* **64**(19-20)**:** 2564-2574

Nikko E, Andre B (2007) Evidence for a direct role of the Doa4 deubiquitinating enzyme in protein sorting into the MVB pathway. *Traffic* **8**(5)**:** 566-581

Nilius B, Mahieu F, Karashima Y, Voets T (2007a) Regulation of TRP channels: a voltage-lipid connection. *Biochem Soc Trans* **35**(Pt 1)**:** 105-108

Nilius B, Owsianik G, Voets T, Peters JA (2007b) Transient receptor potential cation channels in disease. *Physiol Rev* **87**(1)**:** 165-217

Payandeh J, Li C, Ramjeesingh M, Poduch E, Bear CE, Pai EF (2008) Probing structure-function relationships and gating mechanisms in the CorA Mg^{2+} transport system. *J Biol Chem* **283**(17)**:** 11721-11733

Payandeh J, Pai EF (2006a) A structural basis for Mg^{2+} homeostasis and the CorA translocation cycle. *EMBO J* **25**(16)**:** 3762-3773

Payandeh J, Pai EF (2006b) Crystallization and preliminary X-ray diffraction analysis of the magnesium transporter CorA. *Acta Crystallogr Sect F Struct Biol Cryst Commun*

62(Pt 2)**:** 148-152

Penner R, Fleig A (2007) The Mg^{2+} and Mg^{2+}-nucleotide-regulated channel-kinase TRPM7. *Handb Exp Pharmacol*(179)**:** 313-328

Pisat NP (2009) Contribution of the Mnr2 Protein to Magnesium Homeostasis in *Saccharomyces cerevisiae*. PhD dissertation, Biology, University of Missouri-St Louis, St Louis.

Pisat NP, Pandey A, Macdiarmid CW (2009) *MNR2* Regulates Intracellular Magnesium Storage in *Saccharomyces cerevisiae*. *Genetics,* in press.

Pizzirusso M, Chang A (2004) Ubiquitin-mediated targeting of a mutant plasma membrane ATPase, Pma1-7, to the endosomal/vacuolar system in yeast. *Mol Biol Cell* **15**(5)**:** 2401-2409

Poschenrieder C, Gunse B, Corrales I, Barcelo J (2008) A glance into aluminum toxicity and resistance in plants. *Science of the Total Environment* **400**(1-3)**:** 356-368

Puig O, Caspary F, Rigaut G, Rutz B, Bouveret E, Bragado-Nilsson E, Wilm M, Seraphin B (2001) The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24**(3)**:** 218-229

Rao IM, Sharp RE, Boyer JS (1987) Leaf Magnesium Alters Photosynthetic Response to Low Water Potentials in Sunflower. *Plant Physiol* **84**(4)**:** 1214-1219

Raths S, Rohrer J, Crausaz F, Riezman H (1993) *end3* and *end4*: two mutants defective in receptor-mediated and fluid phase endocytosis in *Saccharomyces cerevisiae*. *J Cell Biol* **120:** 55-65

Rengasamy P (2006) World salinization with emphasis on Australia. *J Exp Bot* **57**(5)**:** 1017-1023

Roberg KJ, Rowley N, Kaiser CA (1997) Physiological regulation of membrane protein sorting late in the secretory pathway of *Saccharomyces cerevisiae*. *J Cell Biol* **137**(7)**:** 1469-1482

Romani A, Scarpa A (1992) Regulation of cell magnesium. *Arch Biochem Biophys* **298**(1)**:** 1-12

Romani AM, Maguire ME (2002) Hormonal regulation of Mg^{2+} transport and homeostasis in eukaryotic cells. *Biometals* **15**(3)**:** 271-283

Ryan PR, Kinraide TB, Kochian LV (1994) Al^{3+}-Ca^{2+} interactions in aluminium rhizotoxicity. *Planta* **192:** 98-103

Ryazanov AG (2002) Elongation factor-2 kinase and its newly discovered relatives. *FEBS Lett* **514**(1)**:** 26-29

Scarborough GA (1999) Structure and function of the P-type ATPases. *Curr Opin Cell Biol* **11**(4)**:** 517-522

Schindl R, Weghuber J, Romanin C, Schweyen RJ (2007) Mrs2p forms a high conductance Mg^{2+} selective channel in mitochondria. *Biophys J*

Schlingmann KP, Waldegger S, Konrad M, Chubanov V, Gudermann T (2007) TRPM6 and TRPM7--Gatekeepers of human magnesium metabolism. *Biochim Biophys Acta* **1772**(8)**:** 813-821

Schlingmann KP, Weber S, Peters M, Niemann Nejsum L, Vitzthum H, Klingel K, Kratz M, Haddad E, Ristoff E, Dinour D, Syrrou M, Nielsen S, Sassen M, Waldegger S, Seyberth HW, Konrad M (2002) Hypomagnesemia with secondary hypocalcemia is caused by mutations in TRPM6, a new member of the TRPM gene family. *Nat Genet* **31**(2)**:** 166-170

Schmitz C, Deason F, Perraud AL (2007) Molecular components of vertebrate Mg^{2+}-homeostasis regulation. *Magnes Res* **20**(1)**:** 6-18

Schmitz C, Dorovkov MV, Zhao X, Davenport BJ, Ryazanov AG, Perraud AL (2005) The channel kinases TRPM6 and TRPM7 are functionally nonredundant. *J Biol Chem* **280**(45)**:** 37763-37771

Schmitz C, Perraud AL, Fleig A, Scharenberg AM (2004) Dual-function ion channel/protein kinases: novel components of vertebrate magnesium regulatory mechanisms. *Pediatr Res* **55**(5)**:** 734-737

Schmitz C, Perraud AL, Johnson CO, Inabe K, Smith MK, Penner R, Kurosaki T, Fleig A, Scharenberg AM (2003) Regulation of vertebrate cellular Mg^{2+} homeostasis by TRPM7. *Cell* **114**(2)**:** 191-200

Schock I, Gregan J, Steinhauser S, Schweyen R, Brennicke A, Knoop V (2000) A member of a novel *Arabidopsis thaliana* gene family of candidate Mg^{2+} ion transporters complements a yeast mitochondrial group II intron-splicing mutant. *Plant J* **24**(4)**:** 489-501.

Shaul O (2002) Magnesium transport and function in plants: the tip of the iceberg. *Biometals* **15**(3)**:** 309-323

Silva IR, Smyth TJ, Israel DW, Raper CD, Rufty TW (2001a) Magnesium ameliorates aluminum rhizotoxicity in soybean by increasing citric acid production and exudation by roots. *Plant Cell Physiol* **42**(5)**:** 546-554

Silva IR, Smyth TJ, Israel DW, Raper CD, Rufty TW (2001b) Magnesium is more efficient than calcium in alleviating aluminum rhizotoxicity in soybean and its ameliorative effect is not explained by the Gouy-Chapman-Stern model. *Plant Cell Physiol* **42**(5)**:** 538-545

Simm C, Lahner B, Salt D, LeFurgey A, Ingram P, Yandell B, Eide DJ (2007) *Saccharomyces cerevisiae* vacuole in zinc storage and intracellular zinc distribution. *Eukaryot Cell* **6**(7)**:** 1166-1177

Smith CP, Lee WS, Martial S, Knepper M, You G, Sands JM, Hediger MA (1995) Cloning and regulation of expression of the rat kidney urea transporter (rUT2). *J Clin Investigation* **96:** 1556-1563

Smith RL, Banks JL, Snavely MD, Maguire ME (1993) Sequence and topology of the CorA magnesium transport systems of *Salmonella typhimurium* and *Escherichia coli*. Identification of a new class of transport protein. *J Biol Chem* **268**(19)**:** 14071-14080

Smith RL, Maguire ME (1998) Microbial magnesium transport: unusual transporters searching for identity. *Mol Microbiol* **28**(2)**:** 217-226

Snavely MD, Florer JB, Miller CG, Maguire ME (1989) Magnesium transport in *Salmonella typhimurium*: $^{28}Mg^{2+}$ transport by the CorA, MgtA, and MgtB systems. *J Bacteriol* **171**(9)**:** 4761-4766

Snavely MD, Gravina SA, Cheung TT, Miller CG, Maguire ME (1991a) Magnesium transport in *Salmonella typhimurium*. Regulation of mgtA and mgtB expression. *J Biol Chem* **266**(2)**:** 824-829

Snavely MD, Miller CG, Maguire ME (1991b) The *mgtB* Mgt^{2+} transport locus of *Salmonella typhimurium* encodes a P-type ATPase. *J Biol Chem* **266:** 815-823

Soetens O, De Craene JO, Andre B (2001) Ubiquitin is required for sorting to the vacuole of the yeast general amino acid permease, Gap1. *J Biol Chem* **276**(47)**:** 43949-43957

Sontia B, Touyz RM (2007) Magnesium transport in hypertension. *Pathophysiology* **14**(3-4)**:** 205-211

Spinelli SV, Pontel LB, Garcia Vescovi E, Soncini FC (2008) Regulation of magnesium homeostasis in Salmonella: Mg^{2+} targets the mgtA transcript for degradation by RNase E. *FEMS Microbiol Lett* **280**(2)**:** 226-234

Springael JY, Andre B (1998) Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*. *Mol Biol Cell* **9**(6)**:** 1253-1263

Sreedhara A, Cowan JA (2002) Structural and catalytic roles for divalent magnesium in nucleic acid biochemistry. *Biometals* **15**(3)**:** 211-223

Stanbrough M, Magasanik B (1995) Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. *J Bacteriol* **177:** 94-102

Sun OJ, Payn TW (1999) Magnesium nutrition and photosynthesis in *Pinus radiata*: clonal variation and influence of potassium. *Tree Physiology* **19**(8)**:** 535-540

Swaminathan S, Amerik AY, Hochstrasser M (1999) The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast. *Mol Biol Cell* **10**(8)**:** 2583-2594

Tao T, Grulich PF, Kucharski LM, Smith RL, Maguire ME (1998) Magnesium transport in *Salmonella typhimurium*: biphasic magnesium and time dependence of the transcription of the mgtA and mgtCB loci. *Microbiology* **144 (Pt 3):** 655-664

Tao T, Snavely MD, Farr SG, Maguire ME (1995) Magnesium transport in Salmonella typhimurium: mgtA encodes a P-type ATPase and is regulated by Mg^{2+} in a manner similar to that of the mgtB P-type ATPase. *J Bacteriol* **177**(10)**:** 2654-2662

Tashiro M, Konishi M, Iwamoto T, Shigekawa M, Kurihara S (2000) Transport of magnesium by two isoforms of the Na^{+}-Ca^{2+} exchanger expressed in CCL39 fibroblasts. *Pflugers Arch* **440**(6)**:** 819-827

Tiel Groenestege WM, Hoenderop JG, van den Heuvel L, Knoers N, Bindels RJ (2006) The Epithelial Mg^{2+} Channel Transient Receptor Potential Melastatin 6 Is Regulated by Dietary Mg^{2+} Content and Estrogens. *J Am Soc Nephrol*

Touyz RM (2008) Transient receptor potential melastatin 6 and 7 channels, magnesium transport, and vascular biology: implications in hypertension. *Am J Physiol Heart Circ Physiol* **294**(3)**:** H1103-1118

Townsend DE, Esenwine AJ, George J, 3rd, Bross D, Maguire ME, Smith RL (1995) Cloning of the mgtE Mg^{2+} transporter from *Providencia stuartii* and the distribution of mgtE in gram-negative and gram-positive bacteria. *J Bacteriol* **177**(18)**:** 5350-5354

Wachek M, Aichinger MC, Stadler JA, Schweyen RJ, Graschopf A (2006) Oligomerization of the Mg^{2+}-transport proteins Alr1p and Alr2p in yeast plasma membrane. *FEBS J* **273**(18)**:** 4236-4249

Walder RY, Landau D, Meyer P, Shalev H, Tsolia M, Borochowitz Z, Boettger MB, Beck GE, Englehardt RK, Carmi R, Sheffield VC (2002) Mutation of TRPM6 causes familial hypomagnesemia with secondary hypocalcemia. *Nat Genet* **31**(2)**:** 171-174

Waldron KJ, Rutherford JC, Ford D, Robinson NJ (2009) Metalloproteins and metal sensing. *Nature* **460**(7257)**:** 823-830

Wang CY, Yang P, Shi JD, Purohit S, Guo D, An H, Gu JG, Ling J, Dong Z, She JX

(2004) Molecular cloning and characterization of the mouse Acdp gene family. *BMC Genomics* **5**(1)**:** 7

Wiame JM, Grenson M, Arst HN, Jr. (1985) Nitrogen catabolite repression in yeasts and filamentous fungi. *Adv Microb Physiol* **26:** 1-88

Wiesenberger G, Steinleitner K, Malli R, Graier WF, Vormann J, Schweyen RJ, Stadler JA (2007) Mg^{2+} deprivation elicits rapid Ca^{2+} uptake and activates Ca^{2+}/calcineurin signaling in *Saccharomyces cerevisiae*. *Eukaryot Cell* **6**(4)**:** 592-599

Wiesenberger G, Waldherr M, Schweyen RJ (1992) The nuclear gene *MRS2* is essential for the excision of group II introns from yeast mitochondrial transcripts in vivo. *J Biol Chem* **267**(10)**:** 6963-6969

Wolf FI (2004) TRPM7: channeling the future of cellular magnesium homeostasis? *Sci STKE* **2004**(233)**:** pe23

Xu XZ, Moebius F, Gill DL, Montell C (2001) Regulation of melastatin, a TRP-related protein, through interaction with a cytoplasmic isoform. *Proc Natl Acad Sci U S A* **98**(19)**:** 10692-10697

Yan A, Lennarz WJ (2005) Two oligosaccharyl transferase complexes exist in yeast and associate with two different translocons. *Glycobiology* **15**(12)**:** 1407-1415

Zhou H, Clapham DE (2009) Mammalian MagT1 and TUSC3 are required for cellular magnesium uptake and vertebrate embryonic development. *Proc Natl Acad Sci U S A*